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PRINCIPAL INVESTIGATOR: Bruce S. Bochner
(e-mail: bbochner@welchlink.welch.jhu.edu)

INSTITUTION: Johns Hopkins University School of Medicine

GRANT TITLE: Regulation of Human Eosinophil Adhesion in Allergic Inflammation

AWARD PERIOD: 1 March 1996 - 28 February 1998

OBJECTIVE: To examine how adhesion molecule function contributes to the preferential recruitment of eosinophils that occurs during allergic inflammation.

APPROACH: We hypothesized that the function (avidity) and/or expression of adhesion molecules on the eosinophil surface can be selectively regulated, and therefore can preferentially affect their adhesion and migration. We also reasoned that processes which activate eosinophil responses but not other leukocyte responses in vitro have a much higher likelihood to be of relevance to eosinophil-selective recruitment in vivo in allergic diseases. Studies based upon these principles compared the expression and function of integrins on human eosinophils to those on neutrophils. Flow cytometry was used to compare the expression of various beta 1 and beta 2 integrins and integrin activation epitopes recognized by monoclonal antibodies on eosinophils and neutrophils under various conditions, such as after exposure to certain drugs or relevant cytokines and/or chemokines in vitro. Functional consequences of any observed changes were examined directly in adhesion- and migration-based assays in vitro and in vivo.

ACCOMPLISHMENTS (throughout award period): We have completed in vitro studies characterizing the ability of IL-5, RANTES, tyrosine kinase inhibitors and beta 1 integrin-activating antibodies to alter integrin-mediated eosinophil adhesion to various substrates in vitro. Results of some of these studies are now published in the Journal of Allergy and Clinical Immunology. To better examine how these and other agents affect eosinophil migration, we are in the process of characterizing eosinophil chemotactic responses to a variety of C-C chemokines, and how exposure to these chemokines alters integrin function. Exciting preliminary data, to be submitted this fall in abstract form and soon thereafter for publication, indicate that C-C chemokines, especially those that act via the CCR3 receptor on eosinophils, have the ability to promote cell migration by reducing adhesion mediated through their surface beta 1 integrins.

Subsequent studies have focused on the regulation of beta 2 integrin function in eosinophils. The beta 2 family of integrins, CD11a, CD11b, CD11c, and alpha d, are expressed on most leukocytes. We have determined that the newest member of this family, alpha d, is expressed on human eosinophils in peripheral blood and at higher levels on eosinophils in late-phase allergen challenge BAL fluid. Surface expression on eosinophils can be upregulated within minutes by phorbol ester or calcium ionophore A23187. Culture of eosinophils with IL-5 (interleukin-5) leads to a 2-4 fold increase in alpha d levels by 3-7 days without a change in alpha 4 integrin expression. Regarding alpha d/beta 2 ligands, in both freshly isolated and IL-5 cultured eosinophils, as well as alpha d/beta 2 transfected CHO (chinese hamster ovarian) cells, alpha d/beta 2 can function as a ligand for VCAM-1 (vascular cell adhesion molecule-1, CD106). This conclusion is based in part on the ability of monoclonal antibodies to alpha d, beta 2, or VCAM-1 to block cell attachment in

adhesion assays. More specifically, adhesion to VCAM-1 appears to be primarily alpha 4 integrin-dependent in fresh eosinophils, with a small alpha d integrin-dependent component, while adhesion of IL-5 cultured eosinophils to VCAM-1 is equally dependent on alpha 4 and alpha d integrins. Based on the ability of a VCAM-1 blocking antibody to inhibit alpha d/beta 2-dependent CHO cell adhesion, this interaction appears to occur in the first domain of VCAM-1. These data suggest that alpha d/beta 2 is an alternative ligand for the first domain of VCAM-1, and may play a role in eosinophil adhesion to VCAM-1 in states of chronic inflammation. Portions of this work have appeared in abstract form, and a manuscript has been submitted.

With respect to in vivo human studies, we have continued to examine expression of beta 1 and beta 2 integrins and integrin activation epitopes on eosinophils and neutrophils obtained by bronchoalveolar lavage following endobronchial allergen challenge, and are comparing the expression and activation state of these BAL cells to that of their peripheral blood counterparts. These are slow, long term studies, and are being combined with studies of cytokines and chemokines in an attempt to correlate histologic and cytologic inflammatory changes that occur after allergen challenge. While no publications have resulted as yet, it is anticipated that this work will require at least one more year before a manuscript can be written.

Regarding the proposed experiments to use intravital video microscopy to study effects of chemokines and integrin activation on leukocyte rolling and adhesion in the rat mesentery, our initial efforts have established a reliable model for the study of rat leukocyte rolling and adhesion, and have unexpectedly identified expression and function of alpha 4 integrins (probably both alpha 4/beta 7 and alpha 4/beta 1) on rat neutrophils. Several aspects of this work have now been published, with another paper submitted. Unfortunately, our extensive attempts to establish intravital fluoresence microscopy assays in which labeled human neutrophils or eosinophils are infused into the rat and then followed as they move through the rat mesentery have proven unsuccessful due to the fact that too few cells actually travel through the mesenteric vessel under observation. Alternative in vitro assays, however, should still permit us to test our hypotheses as originally proposed. Indeed, we have successfully established an in vitro parallel plate flow chamber system using video microscopy to examine integrin- and selectin-dependent adhesion of human eosinophils under controlled flow conditions, and regulation of rolling adhesion by chemokines and other agents found to alter integrin function. This new technology will be critical for our future research endeavors, and has been included in pending grant proposals.

Finally, during the two year funding period of this award, the principal investigator authored two separate chapters in premiere text books on allergic diseases covering the topic of cell adhesion, and edited another book entitled "Adhesion Molecules in Allergic Diseases".

<u>CONCLUSIONS</u>: A key aspect of the inflammation responsible for asthma is the selective influx and activation of inflammatory leukocytes, especially eosinophils, in the airways and adjoining tissues. As a result of our work and that of other laboratories, it is our conclusion that specific cytokines and chemokines act to facilitate eosinophil recruitment to the lung by altering their patterns of adhesion and migration. Specifically, we believe that these factors can cause changes in both expression and function of cell adhesion molecules. Our work suggests that the integrins alpha 4/beta 1 and alpha d/beta 2 are important for eosinophil adhesion to endothelial counterligands such as VCAM-1. Another conclusion is that C-C chemokines can contribute to selective accumulation of eosinophils by altering the function of their cell-surface integrins. Finally, while animal models can be useful to explore certain mechanisms of disease that cannot be examined in humans, our results also show that for studies of alpha 4/beta 1 integrins, the rat may not be an appropriate species

for some studies because unlike what is seen in humans and other animals, rat neutrophils express alpha 4/beta 1 integrins.

SIGNIFICANCE: Eosinophils are felt to be responsible for many of the pathophysiological abnormalities in chronic allergic diseases such as asthma. Our studies should provide information on which adhesion molecules are selectively activated to promote eosinophil adhesion and migration, and the mechanisms by which this regulation occurs. By identifying and characterizing the adhesion molecules responsible for eosinophil recruitment, and the cytokines and chemokines that regulate the expression and function of these adhesion molecules, our studies may lead to new therapeutic approaches for the treatment of allergic diseases.

PATENT INFORMATION: No patents resulted from this work.

AWARD INFORMATION: Earlier this year, Dr. Bochner was elected to membership in the American Society for Clinical Investigation. Dr. Bochner's application for promotion to Professor of Medicine is pending. Dr. Davenpeck, a co-investigator on this grant, was promoted to Instructor in Medicine.

Finally, it should be noted that the above-mentioned studies, funded in part through this award, has enabled the prinicpal investigator to apply for additional grant funding. He has just recently received five years of support through an RO1 grant application funded by the National Institutes of Health entitled "Integrins and Chemokines in Allergic Cell Recruitment". A second RO1 proposal, entitled "Glycolipid E-selectin Ligands on Human Granulocytes," is currently under review.

PUBLICATIONS AND ABSTRACTS (for total award period):

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2. Davenpeck, K. L., S. A. Sterbinsky and B. S. Bochner. 1998. Rat neutrophils express α4 and β1 integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Blood 91:2341-2346.

3. Davenpeck, K. L., D. A. Steeber, T. F. Tedder and B. S. Bochner. 1997. P- and L-selectin mediate distinct but overlapping functions in endotoxin-induced leukocyte-endothelial interactions in the rat mesentery. J. Immunol. 159:1977-1986.

4. Grayson, M. H., M. Van der Vieren, W. M. Gallatin, P. A. Hoffman and B. S. Bochner. 1997. Expression of a novel ß2 integrin (αdβ2) on human leukocytes and mast cells. J. Allergy Clin. Immunol. 99:S386 (abstr.).

5. Grayson, M. H., M. Van der Vieren, W. M. Gallatin, P. A. Hoffman and B. S. Bochner. 1998. αdβ2 integrin is an alternative ligand for VCAM-1. J. Allergy Clin. Immunol. 101:S51 (abstr.).

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7. Bochner, B. S. and R. P. Schleimer. 1997. Endothelial cells and cell adhesion. In: Allergy, second edition. A.P. Kaplan, editor. W.B. Saunders, Philadelphia, pp. 251-276.

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9. Grayson, M. H., Van der Vieren, M., Sterbinsky, S. A., Gallatin, W. M., Hoffman, P. A., Staunton, D., and Bochner, B. S. $\alpha d\beta 2$ integrin is expressed on human eosinophils and functions as an alternative ligand for VCAM-1. (J. Exp. Med., submitted)

10. Davenpeck, K. L., Zagorski, J., Schleimer, R. P., and Bochner, B. S. Lipopolysaccharide-induced leukocyte rolling and adhesion in the rat mesenteric microcirculation: regulation by glucocorticoids and role of cytokines. (J. Immunol., submitted)

Regulation of α_4 integrin–mediated adhesion of human eosinophils to fibronectin and vascular cell adhesion molecule-1

Kenji Matsumoto, PhD,[a] Sherry A. Sterbinsky, MSB,[a] Carol A. Bickel, MS,[a] David F. H. Zhou, PhD,[b] Nicholas L. Kovach, MD,[c] and Bruce S. Bochner, MD[a]
Baltimore and Rockville, Md., and Seattle, Wash.

Background: Eosinophils selectively accumulate at sites of allergic inflammation. Their recruitment is dependent on both the expression and functional activity of cell adhesion molecules. How the functional activity of cell adhesion molecules on eosinophils is regulated is poorly understood.

Objective: Our objective was to examine the functional activity of α_4 integrins on human eosinophils and its regulation by various agents.

Methods: Function of α_4 integrins on human eosinophils was examined by testing adhesion to immobilized fibronectin and vascular cell adhesion molecule-1 (VCAM-1) in the presence or absence of a monoclonal antibody (mAb) (8A2) that activates β_1 integrin function.

Results: Spontaneous eosinophil adhesion to VCAM-1 was enhanced by 8A2, but adhesion to fibronectin could only be detected in the presence of 8A2. Concentrations of 8A2 that were approximately 100-fold less than saturating induced maximal eosinophil adhesion. Adhesion to VCAM-1 in the presence of 8A2 was effectively inhibited by α_4 and β_1 integrin mAbs; β_7 mAb had partial inhibitory activity. Connecting segment-1 peptide and α_4 mAb blocked 8A2-dependent fibronectin binding; β_1, β_2, and β_7 integrin mAbs had partial inhibitory activity. Eosinophils obtained from bronchoalveolar lavage fluids and blood eosinophils stimulated with IL-5, platelet-activating factor, or RANTES displayed increased β_2 integrin–dependent, not α_4 integrin–dependent, attachment. Spontaneous adhesion of eosinophils to VCAM-1 was significantly reduced by the tyrosine kinase inhibitor tyrphostin B46 (inhibitory concentration of 50% ≅ 20 μmol/L); this effect was reversed by 8A2.

Conclusions: The functional activity of integrins on eosinophils can be positively and negatively regulated. Altered integrin avidity may influence eosinophil recruitment in vivo. (J Allergy Clin Immunol 1997;99:648-56.)

Key words: Eosinophil, adhesion, integrin, fibronectin, vascular cell adhesion molecule-1, integrin function

From [a]the Johns Hopkins Asthma and Allergy Center, Baltimore; [b]Otsuka America Pharmaceutical, Ltd., Rockville; and [c]the Department of Medicine, Division of Hematology, University of Washington, Seattle.

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Reprint requests: Bruce S. Bochner, MD, Johns Hopkins Asthma and Allergy Center, Room 3A.62, 5501 Hopkins Bayview Cir., Baltimore, MD 21224-6801.

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Abbreviations used	
BAL:	Bronchoalveolar lavage
BSA:	Bovine serum albumin
CS-1:	Connecting segment-1
IC_{50}:	Inhibitory concentration of 50%
mAb:	Monoclonal antibody
MFI:	Mean fluorescence intensity
PAF:	Platelet-activating factor
PBS:	Phosphate-buffered saline
sVCAM:	Soluble vascular cell adhesion molecule-1
VCAM-1:	Vascular cell adhesion molecule-1

Leukocytes express a wide variety of integrins that allow them to interact with other cells and with extracellular matrix proteins.[1-3] Although some of these adhesion molecules mediate cell trafficking, many can also modulate cellular functions, including induction of proliferation and changes in morphologic features, which are events referred to as outside-in signaling.[3] In particular, eosinophils express $\alpha_4\beta_1$ integrins that are capable of binding to vascular cell adhesion molecule-1 (VCAM-1).[4-9] and attachment to VCAM-1 has been shown to affect eosinophil function.[10] VCAM-1 is selectively expressed on endothelial cells after treatment with IL-4 or IL-13[11-13] and is thought to be a crucial adhesion pathway for the specific accumulation of eosinophils and lymphocytes, but not neutrophils, at allergic inflammatory sites, because neutrophils do not express $\alpha_4\beta_1$ integrin.[14]

The α_4 integrins have also been shown to bind to the alternatively spliced connecting segment-1 (CS-1) region of fibronectin containing the recognition sequence EILDV, although the affinity of binding is lower compared with that for VCAM-1.[15-17] Despite expression of α_4 integrins on eosinophils, it remains a matter of controversy whether they spontaneously attach to fibronectin. Some studies have found augmentation in eosinophil function after incubation on fibronectin,[18-21] whereas other studies failed to detect effects on eosinophils or even their attachment.[22-24] A confounding issue in these studies is the possibility that eosinophil CD11b engagement is occurring[25]; this may be responsible for the observed effects on eosinophil function.[26]

A possible explanation for these inconsistencies may be provided by results of recent studies that suggest that

the activation state of integrins can dramatically influence cell adhesion and function.[2,27-29] For example, ligand binding and certain integrin monoclonal antibodies (mAbs) enhance adhesion, presumably by changing the configuration of the heterodimer,[30,31] whereas inhibitors of signal transduction pathways, such as those involving tyrosine kinases, prevent cell adhesion.[32] Regarding activation of adhesion in eosinophils, one such antibody, mAb 8A2, has been used to demonstrate enhancement of attachment to matrix proteins; adhesion to VCAM-1 was not tested.[23] In this study, however, blocking mAbs for either α_4 or α_5 integrin were found to inhibit attachment to fibronectin; yet subsequent studies failed to detect α_5 integrins on eosinophils,[33,34] raising the possibility of platelet contamination of the eosinophil preparations that can occur.[35] In an attempt to clarify the role of β_1 integrin avidity in eosinophil adhesion, additional studies were done to provide a more detailed analysis of α_4 integrin functional activity on eosinophils. Several mechanisms by which the function of α_4 integrins can be regulated on human eosinophils are described.

METHODS

Reagents

Human serum fibronectin was purchased from the New York Blood Center (New York, N.Y.) and kindly provided by Dr. Shaker Mousa (Dupont-Merck Pharmaceuticals, Wilmington, Del.). The CS-1 portion of fibronectin was synthesized and also generously provided by Dr. Mousa. Human soluble recombinant VCAM-1 (sVCAM) was generated as previously described.[12] Bovine serum albumin (BSA), platelet-activating factor (PAF), and phorbol 12-myristate 13-acetate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Human RANTES was provided as a gift (Dr. Tom Schall, DNAX, Palo Alto, Calif.). Human recombinant IL-5 was purchased from R & D Systems (Minneapolis, Minn.). Fragments of fibronectin containing the RGD domain were purchased from Gibco BRL (Grand Island, N.Y.).

mAbs

The β_1 integrin activating mAb 8A2 (mouse IgG1) was generated as previously described.[30] The following murine IgG1 mAbs were generously provided: CD18 blocking mAb H52[36] (Dr. James Hildreth, Johns Hopkins University School of Medicine); β_1 integrin blocking mAb 33B6[33] (Drs. John Bednarczyk and Bradley McIntyre, University of Texas, Houston); and β_1 integrin activation epitope-detecting mAb 15/7[37] (Dr. Ted Yednock, Athena Neurosciences, So. San Francisco, Calif.). Blocking mouse IgG1 mAb recognizing α_4 (HP2/1), α_v (AMF7), and β_3 (SZ.21) integrins (the latter two expressed on platelets but not eosinophils[38]) were purchased from Immunotech (Westbrook, Maine), a blocking mouse IgG1 mAb recognizing α_5 integrin (P1D6) was purchased from Gibco; and an irrelevant mouse IgG1 control antibody was purchased from Coulter Corporation (Hialeah, Fla.). A rat mAb reacting with human β_1 integrin (Fib504) was provided by Dr. Charles Mackay (Leukosite, Inc., Boston, Mass.).

Isolation of human eosinophils

Eosinophils were purified from peripheral blood of donors with allergies by using density gradient centrifugation and negative selection with immunomagnetic beads as previously described.[39] Purity and viability were determined by Diff-Quik staining (American Scientific Products, McGraw Park, Ill.) of cytocentrifuge preparations (Shandon, Pittsburgh, Pa.) and by erythrocin B dye exclusion (Sigma) and were consistently greater than 97% and 99%, respectively ($n = 20$). For some experiments, eosinophils were purified from bronchoalveolar lavage (BAL) fluid obtained 19 hours after segmental challenge of subjects with allergic asthma with allergen[40] with use of the same purification procedures just described. Purity and viability were 100% and 99%, respectively ($n = 3$).

Culture of Jurkat cells

The Jurkat T lymphocytic cell line, a generous gift of Dr. Vincenzo Casolaro (Johns Hopkins Asthma and Allergy Center), was passaged every 3 to 5 days in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Gibco BRL).

Flow cytometric analysis of cell surface integrin expression

Expression of integrins on eosinophils or Jurkat cells was examined by indirect immunofluorescence and flow cytometry as previously described.[33] Briefly, freshly isolated eosinophils or Jurkat cells were incubated (30 minutes, 4° C) in Dulbecco's phosphate-buffered saline (PBS) solution containing 0.1% BSA (Sigma) and 4 mg/ml human IgG (Sigma) with saturating concentrations of mAb or an equivalent concentration of irrelevant IgG1 control mAb. Cells were washed and then incubated (30 minutes at 4° C in PBS containing 0.1% BSA) with 1:20 to 1:50 dilutions of R-phycoerythrin conjugated $F(ab')_2$ goat anti-mouse IgG antibody (Tago Inc., Burlingame, Calif.). After fixation in 1% paraformaldehyde in PBS, at least 8000 cells were evaluated with use of a Coulter EPICS Profile flow cytometer (Coulter). In some experiments, cells were exposed to various signal transduction inhibitors (see later section) before mAb labeling. Fluorescence intensity was determined on a 3-log scale.

Fibronectin and VCAM-1 adhesion assays

For matrix protein experiments, 96-well microtiter plates (Costar Corp., Cambridge, Mass.) were coated overnight at 4° C with 50 μl aliquots of fibronectin (0.016 to 500 μg/ml) diluted in PBS.[20] In other experiments, 96-well microtiter plates (NUNC Maxi-sorb Immunoplates, PGC Scientific Corp., Gaithersburg, Md.) were coated overnight at 4° with 50 μl aliquots of sVCAM (0.4 to 6 μg/ml) diluted in PBS containing $CaCl_2$, 130 mg/L, and $MgCl_2$, 100 mg/L.[12] For both types of plates, the wells were then blocked by incubation with 100 μl aliquots of PBS containing 3% BSA (heat denatured at 65° C for 1 hour) for at least 1 hour at room temperature to reduce nonspecific adherence to plastic. Control adherence was measured in wells coated with PBS alone and blocked with PBS containing 3% BSA. Wells were washed twice with prewarmed Eagle's minimal essential medium (Gibco) and 50 μl aliquots of ^{51}Cr-labeled eosinophils or Jurkat cells (1.25×10^5 total cells per aliquot) were added to wells in triplicate.[33] Cells were allowed to adhere for up to 4 hours (typically 60 minutes, see text) at 37° C after which nonadherent cells were removed by gentle aspiration and rinsing with prewarmed Eagle's medium. Adherent cells were then lysed with NH_4OH, 1 mol/L, for 25 minutes at room temperature and radioactivity of adherent cell

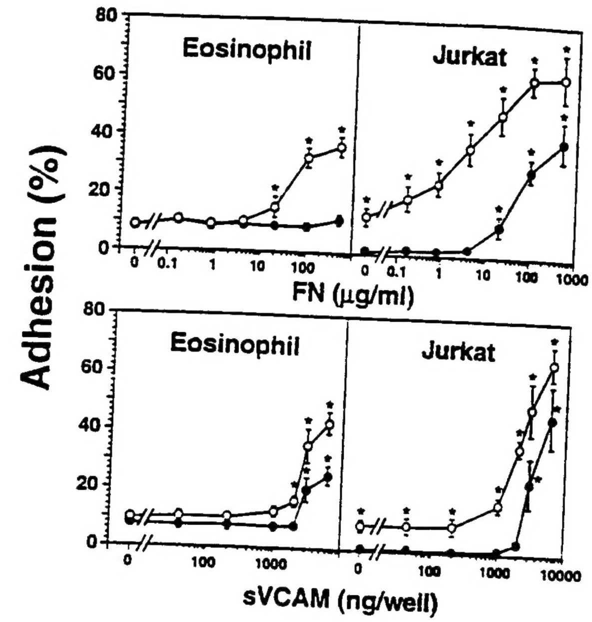

FIG. 1. Comparison of eosinophil and Jurkat cell binding to fibronectin *(FN, upper panels)* and VCAM-1 *(lower panels)* at 60 minutes. In the presence of mAb 8A2 (10 μg/ml, *open circles*), eosinophil binding to fibronectin was detected, and there was significant enhancement of Jurkat cell binding. For VCAM-1, both eosinophils and Jurkat cells demonstrated spontaneous adhesion that was significantly enhanced by mAb 8A2. Jurkat cell adhesion to BSA was also significantly enhanced by mAb 8A2. *Solid circles*, Absence of mAb 8A2; $n = 4$ to 13; $^*p < 0.05$.

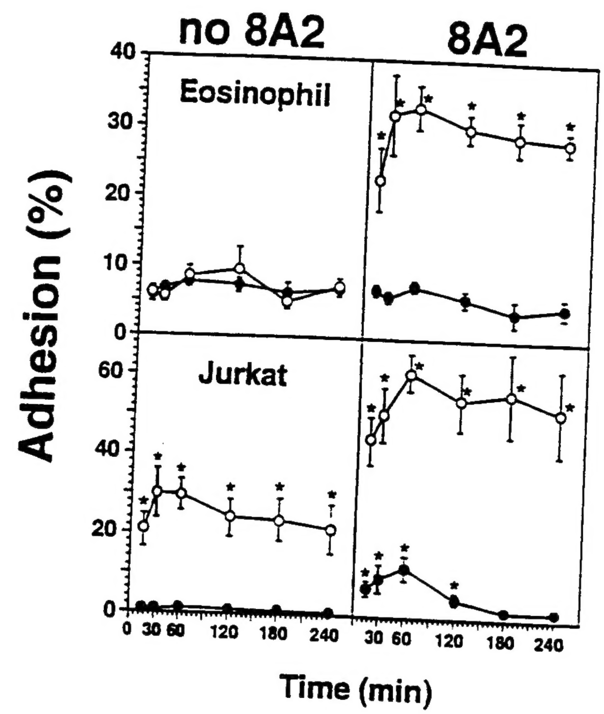

FIG. 2. Kinetics of eosinophil *(upper panels)* and Jurkat cell *(lower panels)* binding to fibronectin (100 μg/ml, *open circles*) or BSA *(closed circles)* in the presence or absence of mAb 8A2 (10 μg/ml). $n = 3$ to 13; $^*p < 0.05$.

lysates was determined with a gamma counter. Percent adherence was calculated by comparing the radioactivity of adherent cell lysates with that of separate 50 μl aliquots of cell suspension. In certain experiments, saturating concentrations of blocking or activating mAbs, various concentrations of CS-1 peptide, or various stimuli such as PAF (10^{-7} mol/L), RANTES (100 ng/ml), phorbol 12-myristate 13-acetate (10 ng/ml), or IL-5 (10 ng/ml) were added to the wells simultaneously with cells and allowed to remain throughout the entire adhesion assay.

For experiments designed to examine potential signal transduction pathways regulating cell adhesion, various pharmacologic inhibitors were used. In these experiments, ^{51}Cr-labeled cells were preincubated for 20 minutes at 37° C with up to 50 μmol/L concentrations of the tyrosine kinase inhibitor tyrphostin B46[41] or the protein kinase C inhibitor staurosporine (up to 50 nmol/L, Sigma) before being added to the wells. In other experiments, cells were preincubated with the G protein inhibitor pertussis toxin (up to 1000 ng/ml, Sigma) for 2 hours and subsequently labeled with ^{51}Cr for adhesion assay as described previously herein.

Statistical analyses

Data are presented as mean plus or minus the standard error of the mean. Statistical significance was determined by *t* test and considered significant at $p < 0.05$.

RESULTS

Eosinophil and Jurkat cell binding to fibronectin and VCAM-1

In initial experiments, eosinophil binding to control BSA-blocked wells was 8.2% ± 1.0% and showed no increase in binding to a wide range of concentrations of immobilized fibronectin (Fig. 1, *upper panels*). To validate the assay and to confirm adequate coating with fibronectin, Jurkat cells were used. Jurkat cell binding to BSA was very low (1.1% ± 0.3%), and significant spontaneous binding to fibronectin was observed at concentrations ≥20 μg/ml (Fig. 1, *upper panels*). In the presence of saturating concentrations (10 μg/ml) of the β_1 integrin activating mAb 8A2, eosinophils showed significant binding to fibronectin at concentrations ranging from 20 to 500 μg/ml. Significant enhancement of Jurkat cell binding was observed at all concentrations of fibronectin tested and with BSA-coated wells.

In contrast to binding to fibronectin, eosinophils showed significant, spontaneous, concentration-dependent binding to VCAM-1 that was maximal at 6 μg/well sVCAM (Fig. 1, *lower panels*). Jurkat cells also showed significant spontaneous binding to VCAM-1 at concentrations ≥2 μg/well (maximum 50.3% ± 9.4%). At each concentration tested, Jurkat cells displayed higher levels of binding than those seen for eosinophils. In the presence of mAb 8A2, eosinophil binding to VCAM-1 (≥2 μg/well) was significantly enhanced. Jurkat cell binding to VCAM-1 was increased at all concentrations of sVCAM tested, as was adhesion to BSA.

Kinetics of eosinophil and Jurkat cell adhesion to fibronectin

To determine whether mAb 8A2 changed the kinetics of cell attachment, eosinophil and Jurkat cell binding to

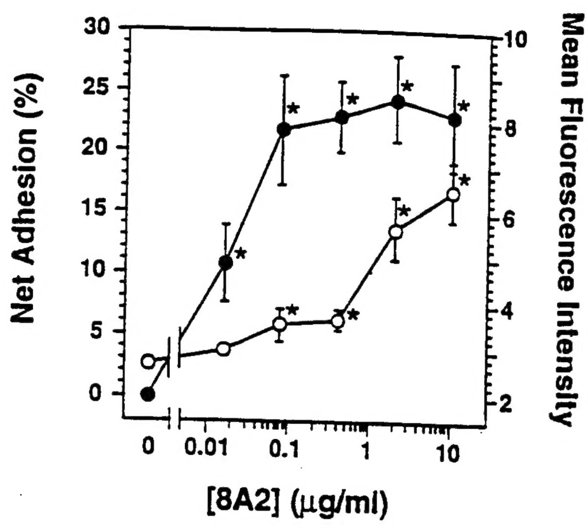

FIG. 3. Titration of binding of mAb 8A2 by indirect immunofluorescence and flow cytometry *(open circles)* and comparison with its effect on eosinophil adhesion to fibronectin (100 μg/ml, 60 minutes, *closed circles*). Control fluorescence intensity with IgG1 control mAb was 2.7 ± 0.1. Values for adhesion to BSA (13% ± 2.6%) were subtracted from those for fibronectin adhesion to yield values for net adhesion. n = 3 to 4; *$p < 0.05$.

fibronectin (100 μg/ml) and BSA was determined at various times ranging from 15 minutes to 4 hours. Throughout this period, eosinophils failed to bind to fibronectin at levels higher than that seen with BSA alone (Fig. 2, *upper panels*). When mAb 8A2 was added at the beginning of the adhesion assay, eosinophils showed rapid and sustained increases in binding to fibronectin that were detectable at 15 minutes and maximal at 30 to 60 minutes. Longer incubation times led to a slight decline in adhesion. In contrast to eosinophils, Jurkat cells again bound spontaneously to fibronectin at all times tested (Fig. 2, *lower panels*). Like eosinophils, Jurkat cell adhesion was maximal at 30 to 60 minutes, then declined slightly at later times. In the presence of mAb 8A2, Jurkat cell binding to fibronectin was augmented but was still maximal at 60 minutes. Augmentation of binding to BSA was also observed at times up to 2 hours.

Titration of mAb 8A2 cell surface binding and effects on adhesion

To determine whether saturating concentrations of mAb 8A2 were needed for optimal augmentation of adhesion, experiments were done in which effects of mAb 8A2 on eosinophil binding to fibronectin were compared with eosinophil surface labeling as measured by indirect immunofluorescence and flow cytometry. As shown in Fig. 3, eosinophil binding to fibronectin in a concentration of 100 μg/ml was significantly enhanced by addition of mAb 8A2 at concentrations as low as 0.0016 μg/ml and was maximally increased at 0.08 μg/ml. This concentration was approximately 2 logs lower than that required for saturable labeling as determined by

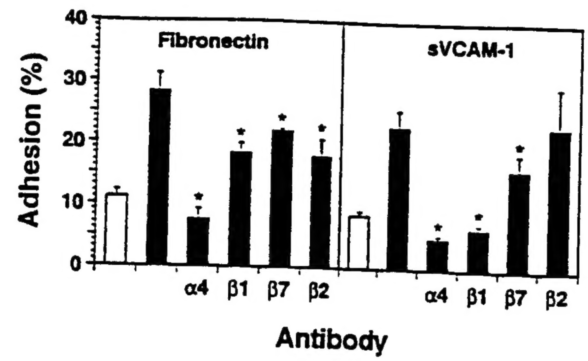

FIG. 4. Effect of integrin blocking mAb on mAb 8A2-induced eosinophil binding to fibronectin and VCAM-1. Values for adhesion to control BSA-coated wells are shown as *open bars*. n = 3 to 8; *$p < 0.05$.

flow cytometry (10 μg/ml). Thus much smaller concentrations of 8A2 mAb were sufficient to induce maximal increases in eosinophil binding to fibronectin.

Effect of integrin function–blocking mAbs on 8A2-induced eosinophil binding to fibronectin and VCAM-1

To examine the specific ligands used by mAb 8A2-treated eosinophils to attach to fibronectin and VCAM-1, adhesion assays were done in the presence and absence of α_4, β_1, β_2, and β_7 integrin blocking mAb. As shown in Fig. 4, eosinophils incubated with mAb 8A2 displayed significant adhesion to both immobilized substrates. Adhesion to VCAM-1 was effectively inhibited by either α_4 or β_1 integrin-specific blocking mAb. Antibody to β_7 also had significant, but less marked, inhibitory activity, whereas β_2 mAb had no effect. Adhesion to fibronectin showed a similar pattern of significant inhibition with α_4, β_1, and β_7 integrin mAb, although the inhibition with β_1 mAb was less complete than that observed in the VCAM-1 adhesion assay. Unlike VCAM-1 adhesion, in the fibronectin adhesion assay the β_2 mAb was as effective as the β_1 mAb in inhibiting adhesion, which suggests that eosinophil attachment was partially β_2 integrin dependent. Indeed, for fibronectin adhesion, a combination of all three β chain mAbs (β_1 + β_2 + β_7) was needed to completely inhibit adhesion (n = 2, data not shown), even though the α_4 mAb by itself blocked well. Unlike findings in previous studies,[23] no effect was seen with an α_5 integrin blocking antibody (P1D6, data not shown), consistent with the reported lack of α_5 integrin expression on eosinophils.[33,34]

Effect of CS-1 peptide on eosinophil and Jurkat cell adhesion to fibronectin

To determine whether adhesion to fibronectin was primarily to the CS-1 domain of the molecule, binding to fibronectin of mAb 8A2-treated eosinophils and untreated Jurkat cells was examined in the presence and absence of soluble CS-1 fragment. As shown in Fig. 5, binding of both cell types was significantly inhibited at

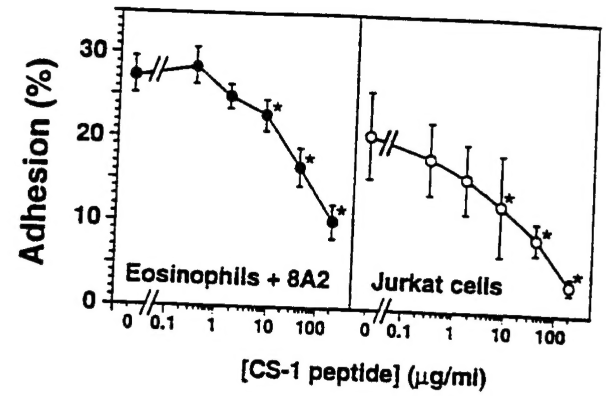

FIG. 5. Effect of CS-1 peptide on mAb 8A2-induced (10 μg/ml) eosinophils and untreated Jurkat cell binding to fibronectin. Adhesion assays were done for 60 minutes. $n = 3$ to 4; $*p < 0.05$.

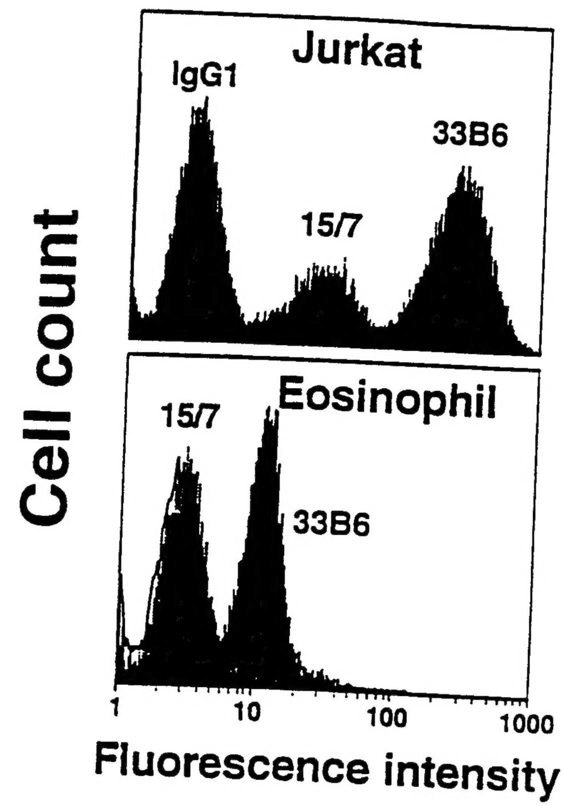

FIG. 6. Expression of β_1 integrin activation epitope detected by mAb 15/7 on eosinophils and Jurkat cells. Eosinophils expressed β_1 integrins (mAb 33B6: mean fluorescence intensity [MFI]: 10.5) with little or no expression of activation epitope detected by mAb 15/7 (MFI: 3.0; IgG control: 3.0). Jurkat cells expressed much higher levels of β_1 integrins (MFI: 120) and the 15/7 activation epitope (MFI: 20). Data are from a single experiment representative of two separate experiments.

concentrations of CS-1 peptide ≥40 μg/ml and approached levels of adhesion seen with BSA. In contrast, addition of similar concentrations of the RGD-containing peptide fragment of fibronectin had no effect on adhesion, nor did CS-1 peptide affect cell binding to VCAM-1 ($n = 2$, data not shown).

Comparison of surface levels of total and activated β_1 integrins and other integrins on eosinophils and Jurkat cells

Because of the higher levels of spontaneous and mAb 8A2-induced adhesion of Jurkat cells compared with that of eosinophils, we determined whether Jurkat cells expressed higher levels of activated β_1 integrins on their surface. To test this hypothesis, both cell types were labeled by indirect immunofluorescence and analyzed by flow cytometry after incubation with mAb recognizing activated β_1 integrin (15/7) or with mAb recognizing β_1 integrins regardless of activation status (33B6).[42] As shown in Fig. 6, Jurkat cells expressed much higher levels of both total and activated β_1 integrins. In fact, eosinophil labeling with mAb 15/7 could barely be distinguished from that seen with the irrelevant control IgG1 mAb, which is consistent with our previous observation that Jurkat cell adhesion to fibronectin and VCAM-1 was more readily demonstrable than that seen with eosinophils. Platelet attachment to eosinophils was not observed, because no binding of mAb to α_5, α_v, or β_3 integrins was ever detected (data not shown).

Effect of eosinophil activation in vitro or in vivo on adhesion to fibronectin

Previous studies have shown that adhesion to endothelium and transendothelial migration are enhanced in eosinophils isolated from late phase BAL fluids or after their exposure to PAF, IL-5, or RANTES.[43-47] To determine whether these conditions also resulted in augmented fibronectin binding, purified BAL fluid eosinophils or peripheral blood eosinophils exposed to various stimuli in vitro were tested for adhesion to a range of fibronectin concentrations. BAL fluid eosinophils displayed higher than normal levels of control adhesion to BSA alone (Fig. 7); this enhanced adhesion was completely blocked by β_2 integrin mAb (data not shown). Adhesion declined as more fibronectin (and presumably less BSA) was used to coat the wells and, as was seen in Fig. 1, mAb 8A2 was required to demonstrate binding, which was detected over a similar range of fibronectin concentrations. As shown in Fig. 8, stimulation of peripheral blood eosinophils with RANTES in vitro had no effect on adhesion, whereas IL-5, and to a lesser extent PAF, increased control adhesion to BSA. As was seen with BAL fluid eosinophils, adhesion declined as higher concentrations of fibronectin were used. The enhancement of adhesion induced by IL-5 and PAF was completely β_2 integrin dependent (data not shown).

Effect of signal transduction inhibitors on Jurkat cell binding to fibronectin and eosinophil and Jurkat cell binding to VCAM-1

Several studies have implicated tyrosine kinases, such as focal adhesion kinase, and serine-threonine kinases, such as protein kinase C, in integrin signal transduction pathways.[32] Therefore studies were done in which phar-

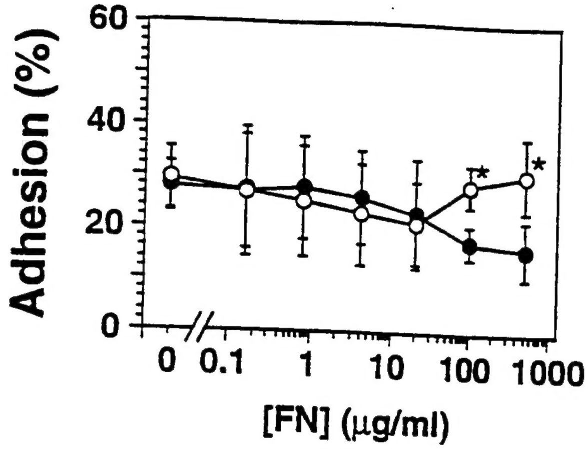

FIG. 7. Adhesion of eosinophils purified from late-phase BAL fluid to fibronectin *(FN)* in the presence *(open circles)* or absence *(closed circles)* of mAb 8A2. $n = 3$ to 5; $^*p < 0.05$.

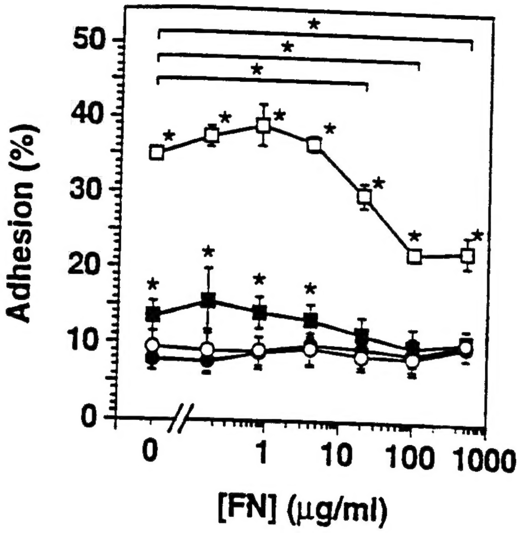

FIG. 8. Effect of IL-5 (10 ng/ml, *open squares*), PAF (10^{-7} mol/L, *closed squares*), and RANTES (100 ng/ml, *open circles*) on eosinophil binding to fibronectin *(FN)*. Adhesion in the absence of stimulus is also shown *(closed circles)*. $n = 3$ to 6; $^*p < 0.05$.

macologic agents that can affect phosphorylation events were tested for their ability to alter adhesion to fibronectin or VCAM-1. Because spontaneous α_4 integrin–dependent adhesion to fibronectin could only be demonstrated with Jurkat cells, the effects of pertussis toxin, staurosporine, and the tyrosine kinase inhibitor tyrphostin B46 were initially examined on these cells. Preincubation with pertussis toxin (up to 1 μg/ml) or staurosporine (up to 50 nmol/L) failed to affect Jurkat cell binding to fibronectin (data not shown). In contrast, Jurkat cell binding to fibronectin was significantly inhibited by tyrphostin B46 in a concentration-dependent manner, with an inhibitory concentration of 50% (IC_{50}) ≅ 20 μmol/L (Fig. 9). This inhibitory effect was completely reversed if mAb 8A2 was present during the adhesion assay. When adhesion of eosinophils and Jurkat cells to VCAM-1 was examined, similar results with tyrphostin B46 were obtained (Fig. 10). Spontaneous eosinophil and Jurkat cell binding to sVCAM (3 μg/well) was significantly inhibited by tyrphostin (IC_{50} ≅ 10 to 40 μmol/L) and the inhibition was completely reversed by mAb 8A2. During the time of preincubation with tyrphostin and subsequent adhesion (80 minutes total), no effect on eosinophil viability was observed (data not shown), consistent with the ability of 8A2 to completely reverse the effects of tyrphostin.

DISCUSSION

Several mechanisms have been identified by which eosinophils are preferentially recruited into allergic inflammatory tissue sites. Certain cytokines and chemokines can selectively activate eosinophil migratory function (e.g., IL-5, RANTES), whereas others (e.g., IL-4, IL-13) promote endothelial cell expression of the adhesion molecule VCAM-1 that acts as a ligand for α_4 integrins expressed on the eosinophil surface. In addition to these pathways, it is now appreciated that the avidity of integrins, not just the total number of molecules expressed, influences cell adhesion and migration[2, 27-29] and that although a particular integrin may have more than one ligand, the avidity for each ligand may differ. It has been known for several years that eosinophils express $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, but little is known about how this cell type regulates α_4 integrin function. In one previous report with eosinophils, a β_1 integrin functional activating mAb that enhances adhesion was used to demonstrate that adhesiveness for matrix proteins including fibronectin could be dramatically potentiated.[23] We have used this same mAb in the present studies to extend the work of Kuijpers et al.[23] by examining the kinetics of this effect, by titrating the effects of this mAb, by demonstrating that the effect is via α_4 integrins and not α_5 integrins, by extending the findings to VCAM-1 adhesion, and by demonstrating that the mAb can reverse the inhibitory effects of tyrosine kinase inhibitors on cell adhesion to VCAM-1 and fibronectin. Adhesion of the human Jurkat T cell line was also examined both to further validate the adhesion assays and to compare α_4 integrin function with that of eosinophils.

As expected, eosinophils and Jurkat cells displayed concentration-dependent adhesion to immobilized VCAM-1. The magnitude of adhesion was enhanced by 8A2, but in marked contrast, spontaneous eosinophil adhesion to fibronectin could not be detected, consistent with some,[23, 24] but not all,[18-21] previous reports. In the presence of 8A2, however, eosinophil attachment to fibronectin was easily demonstrated (Fig. 1).[23] Jurkat cells demonstrated spontaneous adhesion to both fibronectin and VCAM-1, and adhesion to these substrates was enhanced by 8A2. This was consistent with the finding that Jurkat cells express higher levels of activated β_1 integrins (as detected by labeling with mAb 15/7) than eosinophils (Fig. 6). Once cells were exposed

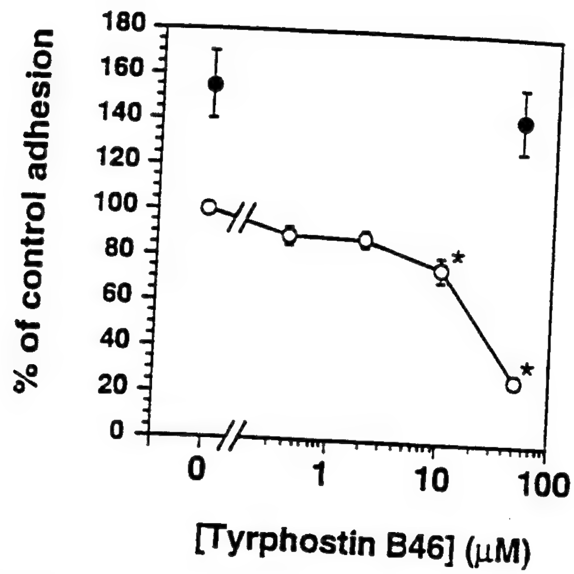

FIG. 9. Effect of tyrphostin B46 on Jurkat cell binding to fibronectin (100 μg/ml) in the presence *(closed circles)* or absence *(open circles)* of mAb 8A2. Control adhesion to fibronectin in absence of mAb 8A2 was 42.4% ± 7.1%. $n = 5$; $*p < 0.05$.

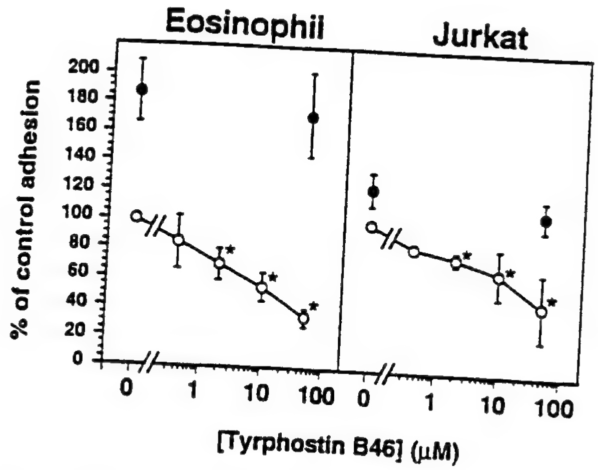

FIG. 10. Effect of tyrphostin B46 on eosinophil *(left panel)* and Jurkat cell *(right panel)* binding to VCAM-1 (3 μg/well) in the presence *(closed circles)* or absence *(open circles)* of mAb 8A2. Values for control adhesion to VCAM-1 for eosinophils and Jurkat cells were 20.3% ± 4.0% and 24.6% ± 8.5%, respectively. $n = 5$; $*p < 0.05$.

to 8A2, the maximal levels of adhesion to VCAM-1 and fibronectin became similar, reaching approximately 40% for eosinophils and 70% for Jurkat cells (Fig. 1), which suggests that maximal activation of β_1 integrin function by 8A2 essentially eliminates any constitutive differences in binding avidity for these two ligands. Despite the observation that 8A2 augmented adhesion, several aspects of the adhesion response remained unaltered. In the presence of 8A2 the concentrations of VCAM-1 and fibronectin that yielded maximal adhesion, and the kinetics of adhesion, were unchanged (Figs. 1 and 2). Interestingly, the amounts of 8A2 needed to optimally augment eosinophil adhesion to fibronectin were approximately 2 logs less than the amounts that were found to be saturating by flow cytometry (Fig. 3), which is consistent with the hypothesis that only a small proportion of β_1 integrin molecules need to be activated to initiate firm cellular attachment.[30, 31, 48]

There are several possible explanations for our inability to detect eosinophil binding to fibronectin in the absence of 8A2. The most plausible explanation is that there simply are too few activated α_4 integrins to mediate binding. Published data with eosinophils that used mAb 15/7 that binds to activated β_1 integrins,[42] as well as comparative data with eosinophils and Jurkat cells in the present manuscript (Fig. 6), support this concept. It is known that binding of α_4 integrins to fibronectin and VCAM-1 occurs through distinct epitopes and that the affinity for the former is less.[4, 5, 49-51] We have made extensive efforts to minimize eosinophil activation during purification, to prevent platelet attachment to eosinophils that can occur during cell isolation,[35] and to establish adhesion assays wherein any β_2 integrin–dependent adhesion responses to blocking proteins are minimized. Evidence of having achieved this is the finding, illustrated in Fig. 4, that eosinophil adhesion to VCAM-1 in the presence of 8A2 was effectively inhibited by mAbs to α_4 and β_1 integrins and less so by an mAb to β_7 integrin, whereas a blocking β_2 mAb had no inhibitory activity. For adhesion to fibronectin, however, blockade with CS-1 peptide or α_4 mAb was most effective, although β_1, β_2, and β_7 integrin mAbs also had partial inhibitory activity, and a combination of all three β chain mAbs ($\beta_1 + \beta_2 + \beta_7$) was needed to completely inhibit adhesion, even though the α_4 mAb by itself blocked well. Our findings regarding the ability of β_7 integrin to serve as a ligand for VCAM-1 and fibronectin are in general agreement with the report of Walsh et al.,[52] although the reason for the differences in integrin use when binding to VCAM-1 versus fibronectin is not clear. Possible explanations may include one or more of the following: (1) slight differences or overlap in the fibronectin recognition epitopes on β_1 integrin bound by the β_1 activating mAb 8A2, which, if already attached to the cell, prevents the β_1 blocking mAb (33B6) from effectively reaching its fibronectin-binding epitope; (2) the possibility that mAb 8A2 binding to eosinophils activates other integrins for binding to fibronectin; and (3) the possibility that α_4 integrin engagement with fibronectin activates adhesion to fibronectin via other integrins. Leukocyte attachment to fibronectin (or blocking proteins such as BSA) has been reported by some laboratories to occur via CD11b.[25, 26, 53] Therefore it is also possible that activation of β_2 integrin pathways can, via outside-in signaling, influence leukocyte activation and promote β_1 integrin–mediated attachment responses. However, when eosinophils were obtained from BAL fluids or peripheral blood eosinophils were stimulated in vitro with IL-5, PAF, or RANTES to display increased β_2 integrin–dependent adhesion, α_4 integrin–

dependent attachment could not be demonstrated (Figs. 7 and 8). These results are consistent with a recent report that demonstrated that IL-5 inhibits expression of a β_1 integrin activation epitope on eosinophils and reduces their attachment to VCAM-1.[42] This report also suggests that conditions that lead to β_2 integrin activation are distinct from those involved in β_1 integrin activation, consistent with a recent report in which C-C chemokines and C5a had differential effects on β_1 and β_2 integrin function in eosinophils.[54] The precise mechanisms by which β_1 and β_2 integrin function might have been reciprocally altered was not the focus of the current studies and will require additional investigation.

Data presented in Figs. 9 and 10 show that spontaneous adhesion of eosinophils to VCAM-1 and of Jurkat cells to both VCAM-1 and fibronectin was significantly reduced by the tyrosine kinase inhibitor tyrphostin B46 ($IC_{50} \cong 20$ μmol/L). The concentrations needed for activity were more that 1 log higher that that reported for inhibition of epidermal growth factor receptor kinase activity ($IC_{50} = 0.7$ μmol/L), but were similar to that reported for inhibition of epidermal growth factor–dependent cell growth ($IC_{50} = 25$ μmol/L).[41] These results are consistent with the emerging concept that integrin-mediated signaling events are associated with tyrosine phosphorylation and formation of focal adhesions.[32] The effect of tyrphostin B46 on eosinophil and Jurkat cell adhesion was reversed by 8A2, which demonstrates that even in the presence of a tyrosine kinase inhibitor, 8A2 has full activity, supporting the hypothesis that the effect of this β_1 integrin–activating antibody is a result of changes in the conformation of the extracellular portion of the molecule. Recently it was reported that a different tyrosine kinase inhibitor, genistein, blocks augmented superoxide anion production that occurs when esoinophils are allowed to attach to immobilized VCAM-1, although this compound failed to affect eosinophil adhesion to VCAM-1.[10] This was confirmed in our hands as well (data not shown). Although other kinases, including the serine-threonine kinase protein kinase C, have also been implicated in integrin signaling, staurosporine did not affect eosinophil attachment. Because both tyrphostins and genistein are often used as tyrosine kinase inhibitors and these compounds have disparate effects on eosinophils, these data suggest the activity of different tyrosine kinases or other unidentified pharmacologic effects are occurring as a result of drug treatment.

Taken together, our results extend those of previous studies by demonstrating several mechanisms that regulate eosinophil α_4 integrin function. Whether a cell increases or decreases β_1 or β_2 integrin function in vivo will likely determine whether the cell will undergo firm endothelial cell attachment, transendothelial migration, strong binding to matrix proteins, or continue to migrate through the tissue. For airway inflammation, it also seems likely that the mechanisms by which eosinophils undergo transepithelial migration to enter the airway lumen will be found to be influenced by the relative avidity of integrins. Data presented so far have identified stimulus-induced downregulation of β_1 integrin function that occurs along with upregulation of β_2 integrin activity. However, whether there exists an opposite pathway by which a physiologic stimulus enhances β_1 integrin function while reducing β_2 integrin activity remains to be elucidated.

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Rat Neutrophils Express α4 and β1 Integrins and Bind to Vascular Cell Adhesion Molecule-1 (VCAM-1) and Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1)

By Kelly L. Davenpeck, Sherry A. Sterbinsky, and Bruce S. Bochner

The α4 integrins, which are constitutively expressed on all human leukocyte subtypes except neutrophils, interact with vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule (MAdCAM-1) on endothelium to mediate selective recruitment of leukocyte subpopulations, other than neutrophils, to sites of inflammation. However, here we report that a different paradigm of leukocyte recruitment may exist in the rat. Flow cytometric analysis of rat neutrophils using a panel of monoclonal antibodies which recognize rat α4 and β1 integrins showed consistent, low levels of expression. Although α4 was expressed at lower levels on neutrophils than all other rat leukocytes, this level of expression was sufficient to mediate significant levels of α4- and β1-dependent neutrophil adhesion to rat and human VCAM-1, and α4-dependent, but β1-independent, adhesion to human MAdCAM-1. These data suggest that rat neutrophils, unlike other species, may use α4 integrins to traffic to sites of inflammation in vivo.

THE INTEGRINS. WHICH mediate leukocyte-endothelial and leukocyte-matrix interactions. are a complex family of heterodimeric glycoproteins consisting of α and β subunit pairs. To date. at least 16 α and eight β subunits have been described and combined to generate over 20 integrin molecules on human cells.[1,2] The α4 integrin subunit, first described by Hemler et al[3] on T-lymphoblastoid cell lines. has been shown to pair with both the β1 and β7 subunits. By virtue of their ability to interact with endothelial expressed ligands. the α4 integrins α4β1 (very late antigen-4 [VLA-4. CD49d/CD29]) and α4β7 (lymphocyte-Peyer's patch adhesion molecule-1 [LPAM-1, CD49d/CD103]), are believed to play a major role in the recruitment of leukocytes during inflammation. In humans, both α4β1. which binds to endothelial vascular cell adhesion molecule-1 (VCAM-1). and α4β7. which interacts with both VCAM-1 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). are constitutively expressed on the surface of eosinophils. basophils. and lymphocytes. but are not detected on neutrophils, whereas monocytes only express α4β1.[4,5] This limited pattern of α4 integrin expression has been theorized to contribute to the selective recruitment of leukocyte subtypes other than neutrophils to sites of inflammation. In addition, the interactions of α4β1 and α4β7 with their endothelial ligands are unique in that unlike the β2 integrins, α4 integrins can mediate both leukocyte rolling and firm adherence to the endothelial surface.[6-9]

Based on the use of blocking antibodies, numerous in vivo studies suggest that α4 integrins can play a role in selective leukocyte recruitment in inflammatory disease processes such as allergic inflammation,[10-12] arthritis,[13] and delayed-type hypersensitivity.[14] Although antibodies to both α4 integrins and VCAM-1 have been used to study selective leukocyte recruitment in various animal models, there has been no thorough analysis performed to establish whether α4 integrin expression on leukocytes in various species is the same as that observed in humans. Though guinea pig and sheep neutrophils do not express α4 integrins,[12,15,16] some studies in rats and mice have unexpectedly found that antibodies to α4 integrins can affect neutrophil recruitment responses and neutrophil-dependent inflammation in vivo.[17,18] Previously, these findings have been attributed to α4 integrin antibody effects on other leukocytes, which in turn may affect neutrophil recruitment. However, Issekutz et al[19] have recently shown that, unlike human neutrophils. rat neutrophils constitutively express low levels of α4 integrins. and that administration of an anti-α4 monoclonal antibody (MoAb), in conjunction with an anti-β2 integrin MoAb. inhibits neutrophil migration into arthritic joints in the rat. Although these findings strongly suggest a role for neutrophil-expressed α4 integrins, the investigators did not confirm neutrophil interaction with the endothelial ligands VCAM-1 or MAdCAM-1.[19] In the present study, we confirm and extend the findings of Issekutz et al[19] by showing that rat neutrophils consistently express α4β1 integrins and use α4β1 integrins to bind VCAM-1, whereas only α4 integrins are used to bind MAdCAM-1 in vitro.

MATERIALS AND METHODS

Rat leukocyte isolation. Whole blood leukocytes and enriched neutrophil populations were isolated from pentobarbital-anesthetized male Sprague-Dawley rats (Charles River Labs Inc. Wilmington. MA and Harlan Sprague Dawley, Indianapolis, IN) weighing 275 to 300 g. EDTA-anticoagulated arterial blood was obtained via cannulation of the right carotid artery. For whole blood leukocytes, a leukocyte-rich buffy coat was obtained by centrifugation at 400g for 20 minutes at 22°C. Contaminating red blood cells (RBC) were removed via hypotonic lysis performed at 4°C. Cell differentials were determined by Diff-Quick staining (Baxter Scientific Products, McGaw, IL) and viability was confirmed by erythrosin B dye exclusion.

Enriched neutrophils populations (polymorph-nuclear leukocyte [PMN]) were obtained via density gradient centrifugation methods, in a manner similar to that described for human neutrophil isolation.[20] In brief, EDTA-anticoagulated whole blood was layered over Percoll

From the Department of Medicine, Division of Clinical Immunology, The Johns Hopkins University School of Medicine. JHAAC. Baltimore, MD.

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Address reprint requests to Bruce S. Bochner, MD, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224.

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(specific gravity, 1.085 g/L) and centrifuged for 20 minutes at 22°C, followed by hypotonic lysis of RBC at 4°C. In preparations in which contaminating lymphocytes made up more than 5% of the cell population, the cells underwent a second centrifugation step over Percoll (specific gravity, 1.085 g/L) to remove these cells. Neutrophil populations were 93.1% ± 0.6% pure with 5.5% ± 0.5% contaminating eosinophils and 1.5% ± 0.3% contaminating lymphocytes ($n < 20$). Cell viability for all flow cytometry and adhesion experiments was greater than 97%.

In addition to isolating neutrophils, mixed populations of rat mononuclear cells (MNC), consisting of lymphocytes and monocytes, were obtained by harvesting the upper layer from the Percoll gradient. These cells were washed twice and subjected to hypotonic lysis to remove any contaminating platelets or RBC. This mononuclear cell population was used as a positive control for analysis of α4 and β1 expression and in VCAM-1 and MAdCAM-1 adhesion assays (see below), and consisted of 7.8% ± 1.5% monocytes and 92.3% ± 1.5% lymphocytes ($n = 6$).

Flow cytometric analysis of leukocyte adhesion molecules. The following α4 integrin MoAbs were purchased and used at the indicated saturating concentrations: TA-2 (immunoglobulin [Ig]G_1, mouse anti-rat, 1 μg/mL; Seikagaku America, Inc, Rockville, MD), MRα4 (IgG_{2b}, mouse anti-rat, 5 μg/mL; Pharmingen, San Diego, CA), and L25 (IgG_1, mouse anti-human, found to cross-react with rat, 5.8 μg/mL; Becton-Dickinson, Mountain View, CA). β1 integrin staining on rat leukocytes was examined using the hamster anti-mouse MoAb Ha2/5 (IgM, 3 μg/mL; Pharmingen) and the hamster anti-mouse MoAb HMβ1-1 (IgG, 3 μg/mL; Pharmingen) found to cross-react with rat. Staining was also attempted with the murine anti-human β7 integrin MoAb ACT-1 (IgG, 1 μg/mL) generously provided by David J. Erle (University of California, San Francisco). Murine anti-rat MoAbs recognizing CD11a (WT.1, IgG_{2a}, 5 μg/mL), CD11b/c (OX-42, IgG_{2a}, 1 μg/mL), CD18 (WT.3, IgG_1, 5 μg/mL), and CD3 (G4.18, IgG_3, 3.1 μg/mL) were also purchased from Pharmingen, and control, nonbinding, isotype-matched mouse IgG_1 and hamster IgM were obtained from Coulter (Hialeah, FL) and Pharmingen, respectively. A mouse anti-human L-selectin MoAb LAM1-116 (IgG_{2a}, 3 μg/mL), cross-reactive with rat, was generously provided by Drs Thomas Tedder and Douglas Steeber (Duke University, Durham, NC).

Labeling of cells for indirect immunofluorescence was performed as described[4] using saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (BioSource International, Camarillo, CA) for all preparations except those in which Ha2/5 or HMβ1-1 was the primary MoAb, in which case an FITC-conjugated goat anti-hamster IgG (H + L) antibody (Jackson Immunoresearch Laboratories, Inc, West Grove, PA) was used. Cells were immediately analyzed unfixed using an EPICS Profile flow cytometer (Coulter Corporation, Hialeah, FL). Monocyte and lymphocyte populations were distinguished via their scatter and CD3:CD11b/c staining characteristics. Neutrophil and eosinophil populations were easily distinguished from each other via their light scatter and α4 integrin staining characteristics (ie, eosinophils have higher forward scatter and higher α4 integrin expression than neutrophils). To examine whether α4 integrin expression could be upregulated by neutrophil activation, enriched neutrophil populations were incubated with either phorbol myristate acetate (PMA; 10 ng/mL), fMLP (10^{-6} mol/L), or C5a (100 ng/mL) for 20 minutes at 37°C before incubation with primary antibodies. Irrelevant isotype-matched control staining with murine IgG_1, IgG_{2a}, or IgG_3, or hamster IgM, typically yielded mean fluorescence values of 2 to 4. Data are presented as fold mean fluorescence above the respective control to facilitate comparisons among various cell types.

Neutrophil labeling with ^{51}Cr and static adhesion assays. For adhesion assays, rat neutrophils, mononuclear cells, and human Jurkat cells were labeled with ^{51}Cr as described for human leukocytes.[20] The Jurkat human T-lymphocytic cell line, a generous gift of Dr Vincenzo Casolaro (Johns Hopkins Asthma and Allergy Center, Baltimore, MD), was used as a control for adhesion assays because these cells are known to constitutively express high levels of α4β1, but they do not express α4β7 ([21] and data not shown). The Jurkat T cells were passaged every 3 to 5 days in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc, Logan, UT), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (GIBCO-BRL). Chinese hamster ovary (CHO) cells and CHO cells stably transfected with rat or human VCAM-1 (known to bind to both human and rat α4[22]; generously supplied by Dr Roy Lobb, Biogen Inc, Cambridge, MA) were grown to confluence as previously described[23] using MEM alpha medium (GIBCO-BRL) supplemented with 10% FBS and methotrexate (500 nmol/L), in 24-well plates for use in static adhesion assays. CHO cells stably transfected with human MAdCAM-1, generously provided by Dr Michael Briskin (LeukoSite, Inc, Cambridge, MA), were grown in a manner identical to VCAM-1–transfected CHO cells except that methotrexate was omitted.

Rat leukocytes and Jurkat T cells were incubated for 30 minutes at 4°C in PAG-Mn buffer (PIPES buffer; 25 mmol/L Piperazine-N, N′-bis-[2-ethanesulfonic acid], 110 mmol/L NaCl, 5 mmol/L KCl [containing 0.003% human serum albumin], 0.1% D-glucose, and 1 mmol/L $MnCl_2$ [Sigma Chemical Co, St Louis, MO]) to enhance α4 avidity.[24] Leukocyte aliquots (100 μL, 2.5×10^5 cells/well) were added in duplicate to each well and allowed to adhere for 10 minutes. All adhesion assays were performed at 4°C to diminish β2 integrin interactions. Nonadherent cells were removed by washing with PAG-Mn. Adherent cells were then lysed with 1 mol/L NH_4OH for 30 minutes, the supernatant removed, and radioactivity counted on a gamma counter. Total counts (ie, total radioactivity) added per well were determined by counting separate aliquots of 2.5×10^5 labeled cells. Percent adhesion was obtained by dividing counts for bound cells by the total counts. In some experiments, cells were preincubated for 30 minutes with blocking antibody to rat α4 (TA-2, 1 μg/mL), β1 (Ha2/5 or HMβ1-1, 3 μg/mL),[4] or CD18 (WT.3, 3 μg/mL) to show the specificity of the adhesion interaction. Preincubation with the human VCAM-1 MoAb 2G7 ($F(ab')_2$, 10 μg/mL)[4] was also used to show adhesion specificity in assays using human VCAM-1–transfected CHO cells. All experiments were performed in duplicate and data are presented as mean adhesion for four to seven individual experiments.

Because enriched neutrophil populations contained approximately 7% contaminating cells, we performed additional experiments to determine if adherent cells were neutrophils or contaminating eosinophils or lymphocytes. In some experiments, non-^{51}Cr–labeled neutrophil preparations were allowed to adhere to rat or human VCAM-1–transfected CHO cells as described. After removal of nonadherent cells, PAG-EDTA (5 mmol/L) was added to the wells for 2 minutes to remove adherent cells. These cells were collected and cell differentials were determined by Diff-Quick staining.

Statistical analysis. All leukocyte adhesion data are presented as mean ± SEM. Data were compared by analysis of variance (ANOVA) using post hoc analysis with Fischer's corrected *t*-test. Probabilities of .05 or less were considered statistically significant.

RESULTS

Rat neutrophils express α4 and β1 integrins. Immunofluorescent staining and flow cytometric analysis were performed on rat whole blood leukocytes and enriched neutrophil populations using a panel of murine anti-α4 MoAbs. Expression of α4 was examined on neutrophils, lymphocytes, monocytes, and eosinophils. Differences in neutrophil and eosinophil scatter in the rat were confirmed in experiments using enriched neutrophil populations in which neutrophils made up approximately 94% of cells, with the remainder being eosinophils. In these experi-

ments two distinct populations, with percentage values corresponding to neutrophils and eosinophils, respectively, could be visualized based on light scatter, and these populations were found to have distinct staining characteristics for α4. These differing scatter characteristics made it possible to independently gate on neutrophils or eosinophils without additional antibody labeling.

Contrary to findings with human neutrophils, rat neutrophils constitutively expressed α4 integrins as confirmed by staining with MoAb TA-2, MRα4, and L25 (Fig 1A and C). Expression of α4 integrins on rat neutrophils was relatively low compared with levels on other cell types, but expression was detectable in all animals examined (Fig 1B). The brightest staining for rat α4 on all cell types was observed with the MoAb TA-2 (Fig 1C). The anti-rat α4 MoAb MRα4 and the anti-human MoAb L25 provided similar levels of staining. Incubation of enriched neutrophil populations with PMA, fMLP, or C5a, at concentrations sufficient to upregulate β2 integrin expression, did not increase expression of α4 as determined by staining with MoAb TA-2 (data not shown).

To determine if neutrophils also expressed β1 integrins, rat neutrophils, and other leukocyte types were first labeled with the anti-β1 MoAb Ha2/5 (Fig 1A). Rat neutrophils consistently showed low, but significant levels of β1 staining (n = 6), as did other leukocyte types (Fig 1A and B). Levels and patterns of staining for β1 on all cell types were similar when the hamster anti-mouse MoAb HMβ1-1 was used (data not shown). Staining for rat β7 was attempted using the murine anti-human β7 MoAb ACT-1, but staining on all rat cell types was negative, implying a lack of MoAb cross-reactivity with rat. As seen in

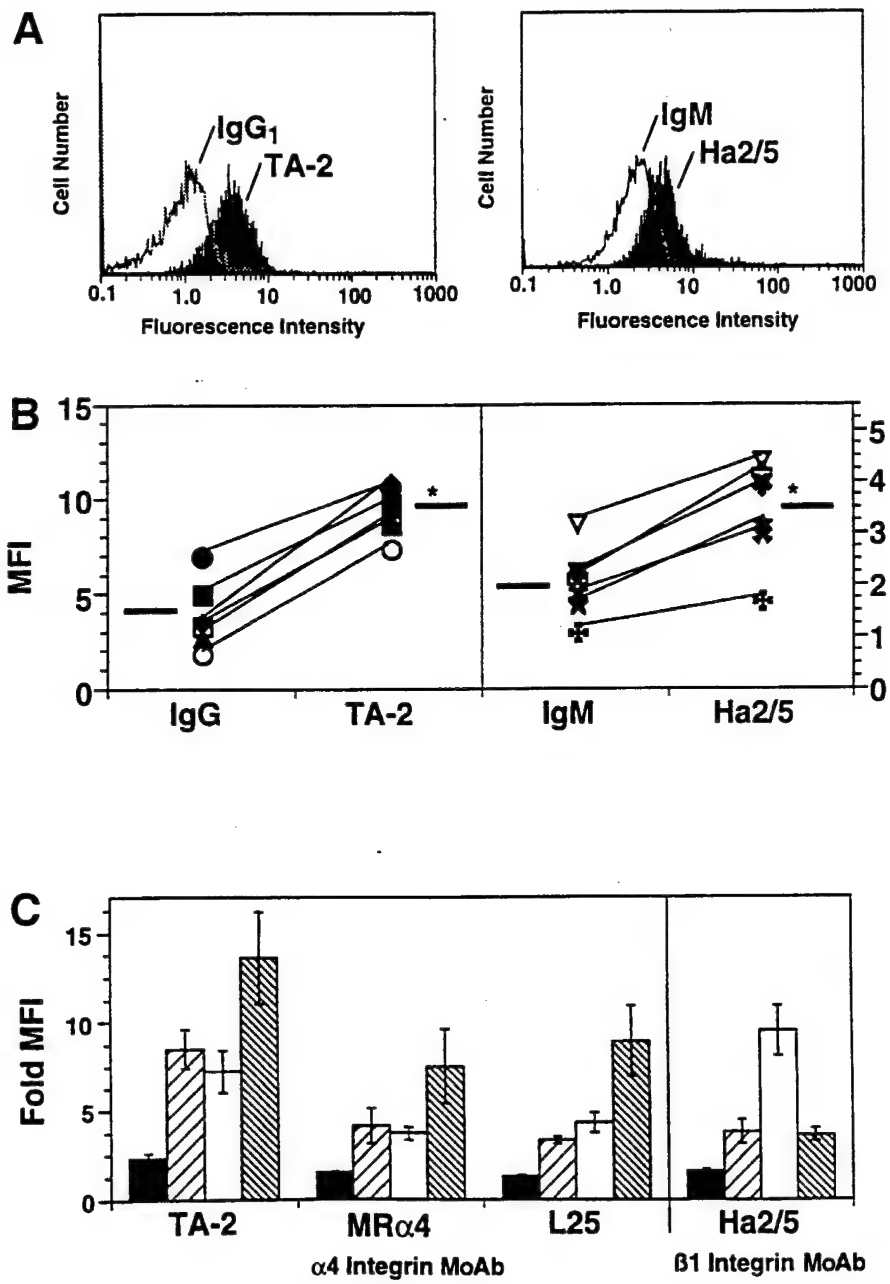

Fig 1. Indirect immunofluorescence and flow cytometric analysis of the surface expression of α4 and β1 integrins on rat leukocytes. (A) Representative histograms of rat neutrophil staining with TA-2 and Ha2/5 as compared with IgG_1 or IgM control, respectively. (B) Actual fluorescence intensity (FI) values for α4 and β1 staining on neutrophils with MoAb TA-2 and Ha2/5 for six rats. *Mean FI values are significantly ($P < .05$) increased over mean FI values for IgG_1 or IgM controls. (C) α4 and β1 expression was examined on neutrophils (PMN, ■), lymphocytes (LYMPH, ▨), monocytes (MONO, □) and eosinophils (EOS, ▧) using the murine anti-rat α4 MoAb TA-2, MRα4, the murine anti-human α4 MoAb L25, and the hamster anti-murine β1 MoAb Ha2/5. Data are presented as fold increase in mean fluorescence intensity (MFI) over IgG or IgM control (n = 6).

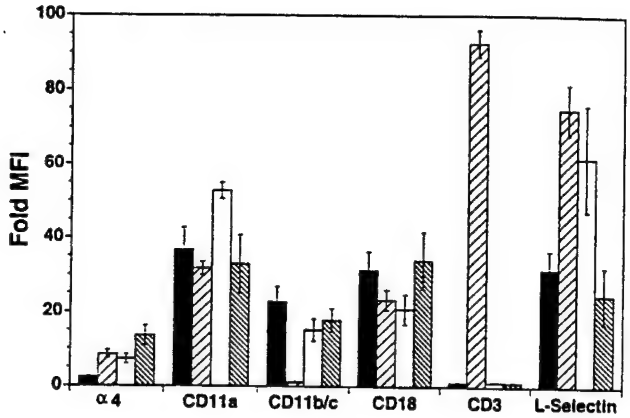

Fig 2. Rat α4 expression as compared with the β2 integrins, CD3, and L-selectin. Indirect immunofluorescence and flow cytometric analysis of the surface expression of α4 (TA-2), CD11a, CD11b/c, CD18, CD3, and L-selectin on rat neutrophils (PMN, ■), lymphocytes (LYMPH, ▨), monocytes (MONO, □), and eosinophils (EOS, ▧). Data are presented as in Fig 1 (n = 5).

Fig 2, the relative amounts of α4 expressed on all rat leukocyte subtypes, as compared with the β2 integrins and L-selectin, was low, even for eosinophils, which showed the strongest α4 integrin staining.

Rat neutrophils adhere to VCAM-1 and MAdCAM-1. To determine if the levels of α4 integrins on neutrophils were sufficient to mediate neutrophil adhesion to the known ligands for α4β1 and α4β7, we first examined rat neutrophil adhesion to rat and human VCAM-1–transfected CHO cells. As shown in Fig 3a, both neutrophils and MNC exhibited significant adherence to rat VCAM-1 CHO cells, as compared with nontransfected CHO cells (eg, for rat neutrophils, 15.8% ± 3.2% *v* 3.4% ± 0.7% adhesion respectively, $P < .01$, n = 7). Affinity of binding was relatively low, because neutrophil adhesion to VCAM-1 was not consistently seen in the absence of Mn^{2+}

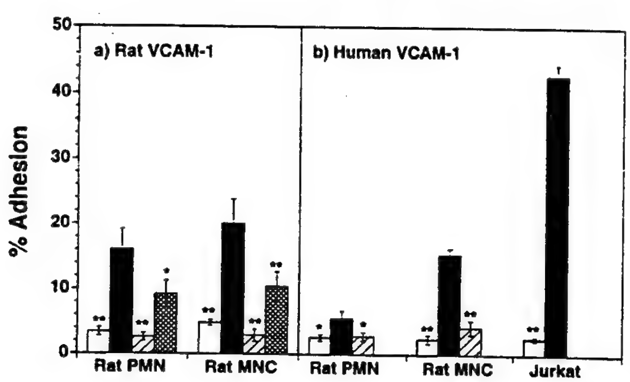

Fig 3. Adhesion of rat neutrophils (PMN) and mononuclear cells (MNC, lymphocytes and monocytes) to untransfected and rat (panel a, n = 7) or human (panel b, n = 6) VCAM-1–transfected CHO cells. Adhesion was tested to nontransfected CHO cells, or CHO cells transfected with VCAM-1 in the presence or absence of α4 MoAb TA-2. Adhesion of rat neutrophils to rat VCAM-1 was also tested in the presence of the β1 antibody Ha2/5. β1 blocking studies were not performed for neutrophil adhesion to human VCAM-1. Data are presented as mean percent adherence ± SEM. *($P < .05$) and **($P < .01$) indicate values significantly different from percent adhesion to VCAM-1–transfected CHO-cells. (□), CHO; (■), VCAM-1 CHO; (▨), VCAM-1 CHO + anti-α4 MoAb; (▩), VCAM-1 CHO + anti-β1 MoAb.

(data not shown). Adhesion of both cell populations to rat VCAM-1 was completely inhibited by incubation of the cells with the mouse anti-rat α4 blocking antibody TA-2 (1 μg/mL) (PMN, 2.6% ± 0.6%; MNC, 2.9% ± 0.8%). Adhesion was only partially inhibited by incubation of the leukocytes with the anti-β1 MoAb Ha2/5 (PMN, 9.2% ± 2.1%; MNC, 10.4% ± 1.7%). Similar results were obtained with the β1 MoAb HMβ1-1 (n = 2, data not shown). The anti-CD18 MoAb WT.3 did not significantly block neutrophil or MNC adhesion to rat VCAM-1 (n = 2, data not shown).

Rat neutrophils and MNC also adhered to human VCAM-1–transfected CHO cells (Fig 3b). Although adhesion was less than that observed with rat VCAM-1 CHO cells (eg, for rat neutrophils, 5.5% ± 1.1% adhesion, n = 6), binding was shown to be α4 specific as MoAb TA-2 completely inhibited adhesion. Adhesion of rat neutrophils and MNC was also completely inhibited by pretreatment of VCAM-1 CHO cells with the mouse anti-human VCAM-1 MoAb 2G7 (n = 6, data not shown). Although rat neutrophils showed consistent adherence to rat and human VCAM-1, neutrophil adherence in both cases was less than that observed for mononuclear cells (Fig 3a and b), consistent with the higher levels of α4 expression on rat MNC. Jurkat cells, which were used as a control cell population, adhered avidly to human VCAM-1 (Fig 3b), consistent with their high levels of α4β1 expression. Because enriched neutrophil populations contained approximately 7% contaminating cells, we performed additional experiments to determine whether the cells adhering to VCAM-1 were neutrophils or contaminating eosinophils or lymphocytes. For both rat and human VCAM-1, neutrophils were found to make up greater than 86% of the adherent cells, with eosinophils making up 10.0% ± 2.5% and lymphocytes 3.0% ± 0.8% (n = 5).

Because β1 integrin blockade only partially inhibited adhesion to VCAM-1, and because we were unable to directly identify β7 integrin expression by flow cytometry, we determined if rat leukocytes could adhere to MAdCAM-1, an α4β7 ligand. As shown in Fig 4, both rat neutrophils and MNC exhibited significant adherence to human MAdCAM-1–transfected CHO cells (eg, neutrophil adhesion 15.9% ± 4.3%), as compared with untransfected CHO cells (4.3% ± 1.2% adhesion; Fig 4). Again, the mouse anti-rat α4 MoAb TA-2 was used to show the α4 specificity of rat neutrophil and MNC adhesion to MAdCAM-1. Adhesion of rat neutrophils and MNC to MAdCAM-1 CHO cells was completely blocked by the addition of MoAb TA-2 to cell preparations. The β1 MoAb Ha2/5 did not have any significant effect on neutrophil adhesion to MAdCAM-1 CHO cells, although it did significantly inhibit MNC adhesion to MAdCAM-1. Similar results were observed for both cell types with MoAb HMβ1-1 (n = 2, data not shown).

DISCUSSION

Previous studies have shown that normal human, guinea pig, and sheep neutrophils do not constitutively express α4 integrins.[4,12,15,16] Although α4 integrins are not constitutively present on human neutrophils, Kubes et al[25] have shown that under certain experimental conditions such as treatment with dihydrocytochalasin B or after in vitro transendothelial migration, human neutrophils can be induced to express α4 integrins and can adhere to stimulated endothelial cells under static and

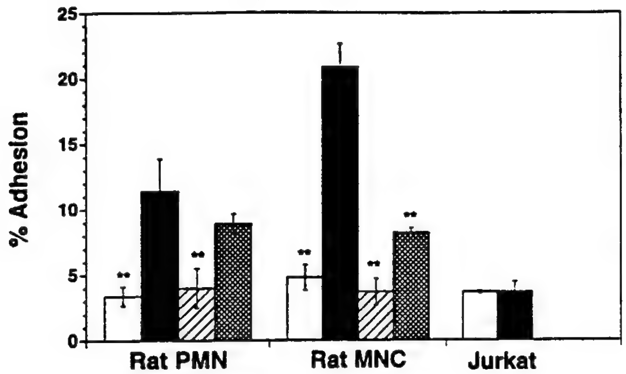

Fig 4. Adhesion of rat neutrophils (PMN, n = 5), mononuclear cells (MNC, n = 5), or Jurkat cells (n = 4) to untransfected and human MAdCAM-1–transfected CHO cells. Adhesion was tested to nontransfected CHO cells, or CHO cells transfected with MAdCAM-1 in the presence or absence of α4 MoAb TA-2 or β1 MoAb Ha2/5. Data are presented as mean percent adherence ± SEM. **(P < .01) indicate values significantly different from percent adhesion to MAdCAM-1–transfected CHO cells. (□), CHO; (■), MAdCAM-1 CHO; (▨), MAdCAM-1 CHO + anti-α4 MoAb; (▩), MAdCAM-1 CHO + anti-β1 MoAb.

flow conditions.[9,25] However, previous data[19] and data presented here indicate that rat neutrophils, unlike other species, constitutively express α4 integrins. Flow cytometric analysis of rat neutrophils using the mouse anti-rat α4 MoAbs TA-2 and MRα4, as well as the mouse anti-human α4 MoAb L25, showed a consistent, low level of α4 expression (eg, 2.3 ± 0.3 mean fold fluorescence above background IgG with TA-2). These data confirm and expand the findings of Issekutz et al[19] in which low levels of α4 expression were shown on the surface of rat neutrophils using a single MoAb TA-2. We show a similar level of α4 expression on neutrophils using MoAb TA-2, as well as with the other MoAbs which bind rat and human α4 integrins. Direct comparison of α4 expressed on neutrophils to that on other rat leukocytes via flow cytometry shows that although they are consistently present, neutrophils express the lowest levels of α4 integrins, with rat eosinophils expressing the highest levels.

In addition, we show low levels of β1 integrin expression on rat neutrophils. Flow cytometric analysis of rat β1 expression using MoAb Ha2/5 revealed consistent, low-level β1 expression on neutrophils, with greater expression on lymphocytes and eosinophils, and the greatest expression on monocytes. However, the partial to minimal inhibitory activity seen with β1 integrin blockade in the VCAM-1 and MAdCAM-1 adhesion assays may suggest the presence of an additional α4 integrin subunit, such as β7. It is possible that neutrophil-expressed α4β7 could account in part for neutrophil adherence to both VCAM-1 and MAdCAM-1, because α4β7 is a ligand for both molecules. Unfortunately, the lack of antibodies which cross-react with rat β7, and the inability to obtain eosinophil-free preparations of neutrophils for immunoprecipitation experiments makes it impossible to determine the exact heterodimeric composition of rat neutrophil α4 integrins at this time. Interestingly, the ability of the anti-β1 MoAb to significantly block MNC adhesion to MAdCAM-1 may suggest that MNC-expressed α4β1 integrins can interact with MAdCAM-1 in the rat. In mouse and human cell systems, α4 integrin interactions with MAdCAM-1 have been seen only with α4β7, not α4β1.

Beyond showing the expression of α4 and β1 on the neutrophil surface, we also showed that neutrophil-expressed α4 integrins can mediate neutrophil, as well as mononuclear cell, adhesion to VCAM-1 and MAdCAM-1 expressed on transfected CHO-cells. Isolated rat neutrophils incubated in Mn^{2+}-containing buffer specifically adhered to both rat and human VCAM-1–transfected CHO cells and MAdCAM-1–transfected CHO cells at 4°C. Neutrophils did not consistently adhere to VCAM-1 or MAdCAM-1 in the absence of Mn^{2+}, suggesting that these cells expressed low levels of activated α4 integrins. This may in part explain differences between our findings and those of Andrew et al[26] in which they were unable to show α4β7-mediated adhesion to VCAM-1 at 4°C. Adhesion to both VCAM-1 and MAdCAM-1 was completely blocked by anti-α4 MoAb TA-2. Our findings that rat neutrophils bind VCAM-1 and MAdCAM-1 in an α4-dependent manner support in vivo data from Issekutz et al[19] which indicate that the MoAb TA-2 may effect neutrophil recruitment in the rat. Although these findings[19] strongly suggest a role for neutrophil-expressed α4, the investigators did not confirm that neutrophil α4 integrins were expressed at sufficient levels to mediate interaction with the endothelial ligands VCAM-1 or MAdCAM-1. However, adhesion data from our studies clearly indicate that the in vivo effect of diminished neutrophil recruitment observed with the administration of MoAb TA-2 is likely the result of antibody blockade of neutrophil interaction with VCAM-1. Furthermore, the ability of α4 integrin MoAb to block rat neutrophil adhesion to MAdCAM-1 suggests that α4 integrin MoAb may also be capable of blocking neutrophil trafficking to the gut. Blocking rat α4 integrins may also inhibit neutrophil interaction with the matrix protein fibronectin, because emigrated rat neutrophil binding to cardiac myocytes has been shown to be α4 integrin and fibronectin dependent.[27]

Studies by Issekutz et al[19] provide the most direct evidence that neutrophil-expressed α4 integrins may be important for neutrophil recruitment in the rat, but earlier data from Mulligan et al,[17] published before α4 integrin identification on rat neutrophils, also support this. In these experiments, the rat α4 antibody TA-2 was found to significantly reduce neutrophil infiltration, changes in lung permeability, and hemorrhage in a model of intrapulmonary IgG deposition. These investigators have previously shown this model of lung injury to be almost exclusively neutrophil mediated, with some role for alveolar macrophages.[28,29] In their discussion of the data the investigators speculate that the effects observed in this model, with the antibody TA-2, may be attributed to a role for α4 in macrophage cytokine release.[17] Although the potential effects of MoAb TA-2 on macrophage function can not be discounted, our findings would suggest that the inhibition of neutrophil infiltration is more likely a direct effect of the antibody on neutrophil interaction with α4 integrin ligands. Examples of α4 MoAb reduction of neutrophil recruitment also exist in the mouse. Chisholm et al[18] found reduced neutrophil-dependent edema with an α4 MoAb treatment in a mouse model of T-cell–dependent contact hypersensitivity. Here the investigators again speculate that the decreased neutrophil recruitment is the result of decreased T-cell infiltration and thus decreased mediator

release. It is likely that these observations are in part correct, but the presence of $\alpha4$ on mouse neutrophils has not been examined, and therefore a direct effect of the $\alpha4$ MoAb on neutrophil recruitment can not be ruled out.

In conclusion, we have shown that rat neutrophils, unlike neutrophils from most other species, constitutively express low levels of functional $\alpha4$ and $\beta1$ integrins. The low level expression of $\alpha4$ integrins can mediate neutrophil binding to both rat and human VCAM-1 as well as human MAdCAM-1. These data show a novel role for $\alpha4$ integrins in rat neutrophil recruitment and suggest that MoAbs reacting with $\alpha4$, $\beta1$, VCAM-1, MAdCAM-1, or perhaps $\beta7$ administered in vivo in rat models of cell recruitment may directly affect neutrophil recruitment.

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P- and L-Selectin Mediate Distinct but Overlapping Functions in Endotoxin-Induced Leukocyte-Endothelial Interactions in the Rat Mesenteric Microcirculation[1]

Kelly L. Davenpeck,* Douglas A. Steeber,† Thomas F. Tedder,† and Bruce S. Bochner[2]*

Endotoxin is a potent stimulus of leukocyte infiltration, but the adhesion-related mechanisms responsible for LPS-induced cell recruitment events in vivo remain poorly characterized. Utilizing intravital microscopy, we examined the role of P- and L-selectin in LPS-induced inflammation. We demonstrated that superfusion of rat mesentery with LPS resulted in significant increases in both leukocyte rolling and adherence, which were maintained for at least 2 h. Pretreatment with a P-selectin neutralizing mAb only partially inhibited LPS-induced leukocyte rolling, but completely inhibited LPS-induced leukocyte adherence throughout the 2-h observation period. Pretreatment with an L-selectin neutralizing mAb dramatically inhibited LPS-induced increases in leukocyte rolling, but unlike the P-selectin mAb did not inhibit leukocyte adhesion. Fucoidin, which blocks both P- and L-selectin function, completely inhibited LPS-induced leukocyte rolling and adhesion. Consistent with previous studies, leukocyte rolling velocities on P-selectin were observed to be far less than velocities observed for leukocytes rolling on L-selectin in vivo. These data suggest that P-selectin plays a role in LPS-induced rolling and is essential for LPS-induced leukocyte adherence, while L-selectin functions in LPS-induced rolling, but not in adhesion. *The Journal of Immunology,* **1997, 159: 1977–1986.**

Lipopolysaccharide (LPS), a component of the outer membrane of most Gram-negative bacteria and referred to as endotoxin, is a highly potent inflammatory agent (1, 2). In the circulation, LPS can precipitate a host of inflammatory events that, in the extreme, result in the multisystem failure associated with Gram-negative sepsis. One of the primary mechanisms by which LPS mediates its inflammatory effects is through activation of the vascular endothelium. Acting through LPS-binding protein and the soluble CD14 receptor molecule (2, 3), LPS induces a multifaceted activation of the vascular endothelial cell, which results in, among other things, up-regulation of endothelial adhesion molecules (4–8), increased cytokine production (9, 10), and increased vascular permeability (11), all changes that contribute to one of the hallmarks of LPS-induced inflammation, leukocyte extravasation into the inflamed tissue (11, 12).

The active movement of leukocytes out of the vasculature into surrounding tissue involves a multistep process resulting from the sequential activation of various adhesion molecules (13–15). The selectin family of adhesion molecules is believed to mediate the earliest phase of leukocyte recruitment, rolling along the endothelium, which serves to tether the unstimulated leukocyte to the activated endothelial surface (16, 17). The integrin and Ig families of adhesion molecules appear to mediate a more firm adherence of the leukocyte and subsequent transendothelial migration (17). LPS has been implicated in altering leukocyte-endothelial interactions, and thus leukocyte extravasation, through a variety of these leukocyte and endothelial adhesion molecules (4–8, 18). For instance, expression of all three selectin molecules can be affected by LPS. Both in vitro and in vivo data demonstrate that LPS can up-regulate expression of P- and E-selectin on endothelium (4, 8, 19, 20). P-selectin, which is stored in the Weibel-Palade bodies of endothelial cells (21), is rapidly translocated (i.e., in 10 min) to the endothelial surface upon stimulation with a variety of preformed mediators, including histamine, thrombin (22, 23), and also LPS (8, 20). Similarly, E-selectin expression can be up-regulated by LPS (4, 19, 24). However, unlike P-selectin, E-selectin expression requires 1 to 4 h to occur (peak expression at 4–6 h), as E-selectin is not stored in the endothelial cell and its expression is therefore dependent on gene transcription and translation (4, 25). LPS can also affect L-selectin expression and L-selectin-mediated leukocyte interactions, although these effects are different on the leukocyte than on the endothelial cell. Like P- and E-selectin, LPS stimulation of endothelial cells up-regulates an as yet unidentified endothelial ligand for L-selectin (5). However, direct stimulation of leukocytes with LPS can down-regulate L-selectin, which is constitutively present on the leukocyte surface (18). Thus, LPS can potentially increase or decrease L-selectin-mediated interactions.

LPS also alters expression of the integrin and Ig families of adhesion molecules (18). The β_2 integrins, which are heterodimers with one of four α subunits (CD11a/CD11b/CD11c/αd) and a common β subunit (CD18) (17), are for the most part constitutively present in modest amounts on unactivated leukocytes, and are rapidly up-regulated in amount and/or function on the cell surface following activation by mediators such as platelet-activating factor (PAF),[3] LTB_4 (17), or LPS (18). LPS also up-regulates a primary endothelial counter-receptor for the β_2 integrins, ICAM-1, a

*Department of Medicine, Division of Clinical Immunology, The Johns Hopkins University School of Medicine, Baltimore, MD 21224; and †Department of Immunology, Duke University Medical Center, Durham, NC 27710

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[2] Address correspondence and reprint requests to Dr. Bruce S. Bochner, The Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. E-mail: bbochner@welchlink.welch.jhu.edu

[3] Abbreviations used in this paper: PAF, platelet-activating factor; MABP, mean arterial blood pressure.

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member of the Ig superfamily (6, 7, 26). ICAM-1 is constitutively expressed at moderate levels and, like E-selectin, requires cytokine- or LPS-induced protein synthesis to reach more effective levels (17). VCAM-1, another member of the Ig superfamily, and the primary endothelial ligand for the β_1 integrin VLA-4 ($\alpha_4\beta_1$), is also up-regulated by LPS (17, 27). The interaction of VLA-4 with VCAM-1 appears to be unique in that this interaction has been demonstrated to mediate both leukocyte rolling and firm adherence (28–30). LPS can also indirectly facilitate endothelial adhesion molecule expression, and thus leukocyte recruitment through stimulation of endothelial cell cytokine production (i.e., IL-1, TNF-α) (9, 11, 12, 31). In vitro stimulation of HUVEC with IL-1 or TNF-α results in up-regulation of ICAM-1, VCAM-1, and E-selectin (32, 33).

Thus, LPS has been demonstrated to affect the expression of many of the leukocyte and endothelial adhesion molecules involved in leukocyte recruitment. Although LPS can induce increased expression of many of these adhesion molecules, the role of each molecule during in vivo LPS-induced leukocyte recruitment has not been established. Many of the in vivo studies examining the role of leukocyte and endothelial adhesion molecules in LPS-induced leukocyte recruitment have focused on the interactions between β_2 integrins and ICAM-1 (34–37), while very few have examined the role of selectins. Those selectin studies that do exist examine leukocyte recruitment following several hours of LPS stimulation (38, 39), while in vitro data indicate that LPS can rapidly (i.e., within minutes) alter the expression of at least two of the selectin molecules (8, 18). In the present study, we have utilized a model of rat intravital microscopy to directly examine the role of selectins in rapid, LPS-induced changes in leukocyte-endothelial interactions in vivo. We report that LPS stimulation of the rat mesenteric microcirculation results in rapid induction of leukocyte rolling and adhesion. These events are shown to be mediated by P- and L-selectin, and important biologic differences are demonstrated.

Materials and Methods

Rat mesenteric intravital microscopy

In accordance with an animal research protocol approved by The Johns Hopkins University Animal Care and Use Committee, male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, and Harlan Sprague Dawley, Indianapolis, IN) weighing 275 to 300 g were anesthetized with sodium pentobarbital (35 mg/kg) injected i.p., and the trachea was cannulated to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in one carotid artery to monitor mean arterial blood pressure (MABP), and a second catheter was placed in the opposite external jugular vein for i.v. infusions. MABP was recorded on a Grass Model 7 oscillographic recorder using Statham P23AC pressure transducers (Gould, Cleveland, OH). The abdominal cavity was opened via a midline laparotomy, and a loop of ileal mesentery was exteriorized through the midline incision and placed in a chamber for intravital microscopic observation of the mesenteric microcirculation. The mesentery was draped over a Plexiglas pedestal in the superfusion chamber, and the ileum was secured for stabilization of the viewing field. The ileum and mesentery were superfused throughout the experiment with a modified Krebs-Henseleit solution (in mM: 118 NaCl, 4.74 KCl, 2.45 $CaCl_2$, 1.19 KH_2PO_4, 1.19 $MgSO_4$, and 12.5 $NaHCO_3$) (Sigma Chemical Co., St. Louis, MO) heated to 37°C and bubbled with 95% N_2 and 5% CO_2. A Zeiss Axioskop fixed stage upright microscope was used for observation of the mesenteric microcirculation. The image was projected by a high resolution CCD camera (Hamamatsu, Japan) to a black and white high resolution monitor, and the image was recorded with a videocassette recorder (Sony Corp. of America, Park Ridge, NJ). RBC velocity was determined on-line using an optical Doppler velocimeter (40) (Microcirculation Research Institute, College Station, TX).

Mean venular diameter, numbers of rolling and adherent leukocytes, as well as leukocyte rolling velocity were determined off-line by playback of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity slower than that of red cells. The rolling rate (i.e., leukocyte flux) was expressed as the number of cells moving past a fixed point per minute. Leukocyte rolling velocity was determined by measuring the time required for a leukocyte to travel 50 μm along the venular endothelium. The velocity for each time point represents the average velocity of 10 leukocytes per recording and was expressed in micrometers per second. A leukocyte was determined to be adherent if it remained stationary for >30 s. Adherence was expressed as number of leukocytes/100 μm of vessel. Venular wall shear rate (γ) was calculated based on RBC velocity and venular diameter using the formula $\gamma = 8\ (V_{mean}/D)$, in which V_{mean} is the mean RBC velocity (i.e., center line velocity/1.6) and D is mean venular diameter (41).

Experimental protocol

Following stabilization of the mesentery, a 20- to 35-μm-diameter postcapillary venule was chosen for observation. A baseline or control recording of 2-min duration was made, and the tissue was then allowed to stabilize for 30 min. If leukocyte rolling or adhesion was observed to increase during this period, the experiment was terminated. Following the 30-min stabilization period, a second video recording (time 0) was made to establish basal values for leukocyte rolling and adherence, and leukocyte rolling velocities. To minimize the influence of preactivation of the tissue, only vessels in which leukocyte rolling was ≤30 cells/min and adhesion ≤3 cells/100 μm of venular endothelium were utilized for study.

In initial studies, the mesentery was superfused with 0.1 to 1 μg/ml of LPS (from *Escherichia coli* serotype 0127:B8, lot 63H4010; Sigma Chemical Co.) in modified Krebs-Henseleit solution for 120 min. LPS superfusion was initiated immediately following the 0-min video recording, and then subsequent 2-min recordings were made at 30, 60, 90, and 120 min after initiation of superfusion for determination of leukocyte rolling and adherence, and leukocyte rolling velocity. Changes in leukocyte-endothelial interactions were compared with leukocyte parameters in a group of sham or buffer control animals in which the surgical procedure and tissue setup were identical to LPS-treated animals, but the mesentery was superfused with only Krebs-Henseleit buffer throughout. Arterial blood samples (100 μl) were obtained at each of the above time points, and circulating total white blood cell numbers were determined by light-microscopic counting (Unopette, Test 5856; Becton Dickinson, Rutherford, NJ). Whole blood smears for determination of leukocyte differentials were also made at baseline, 0, and 120 min. Cell differentials were determined by Diff-Quik staining (Shandon, Pittsburgh, PA).

To determine whether the changes in leukocyte-endothelial interaction observed with LPS from *E. coli* were specific for this bacterial serotype, additional experiments were performed utilizing LPS derived from other bacteria. As the most consistent increases in leukocyte rolling and adherence were observed with 1 μg/ml of LPS from *E. coli* (Fig. 1), this concentration was utilized to examine the effects of LPS derived from *Pseudomonas aeruginosa* (serotype 10, lot 87F4009; Sigma Chemical Co.) and *Salmonella minnesota* (lot 89F4007; Sigma Chemical Co.) on leukocyte-endothelial interaction in the rat mesentery. In these experiments, LPS was superfused over the mesentery, and changes in leukocyte-endothelial interaction were measured, as described for *E. coli*-derived LPS.

In all subsequent studies examining the function of selectins in LPS-induced leukocyte-endothelial interactions, 1 μg/ml of *E. coli*-derived LPS was utilized to stimulate the mesenteric tissue. To antagonize adhesion, mAb that block P- or L-selectin function, their isotype-matched controls, or fucoidin were administered i.v. in PBS (300 μl) 10 min before initiation of LPS superfusion. The murine anti-human P-selectin mAb PB1.3 (IgG1; cross-reactive with rat P-selectin; Cytel Corp., San Diego, CA) was given at a dose of 1 mg/kg (42). The mAb 1E6 (IgG1, mouse anti-human LFA-3, CD58), generously supplied by Dr. Roy Lobb (Biogen, Cambridge, MA (43)), was utilized as an irrelevant isotype-matched control for PB1.3 and was also administered at a dose of 1 mg/kg. The murine anti-L-selectin blocking mAb LAM1-116 (IgG2a) and another binding, but nonblocking L-selectin mAb LAM1-118[4] were administered at doses of 100 μg/rat. Higher doses of these mAb were not utilized, as they resulted in increased leukocyte adhesion in the mesenteric microcirculation. Because we could not obtain a mAb that reacts with rat E-selectin, we utilized fucoidin, an algae-derived polysaccharide containing fucose and fucose 4-sulfate polymer (Sigma Chemical Co.) that has been demonstrated to bind to and block the function of both P- and L-selectin, but not E-selectin (16, 44) to determine whether there would be any residual rolling or adhesion. Fucoidin was administered at a dose of 5 mg/kg 10 min before LPS superfusion; a second dose was administered 60 min later. The second dose of fucoidin

[4] D. A. Steeber, P. Engel, A. S. Miller, M. P. Sheetz, and T. F. Tedder. Ligation of L-selectin through conserved regions within the lectin domain activates signal transduction pathways and integrin function in human, mouse, and rat leukocytes. *Submitted for publication.*

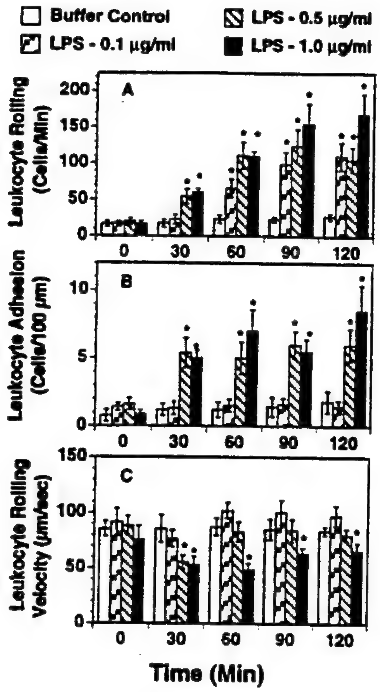

FIGURE 1. Leukocyte rolling (*A*), adhesion (*B*), and rolling velocity (*C*) in postcapillary venules from buffer control and LPS-treated rats. A single loop of mesentery was superfused with LPS in Krebs-Henseleit buffer at concentrations of 0.1, 0.5, and 1 μg/ml. Leukocyte rolling, adhesion, and rolling velocity were quantified at baseline (0 min) and at 30, 60, 90, and 120 min after initiation of LPS superfusion. Bar height represents mean values ($n = 6$ for all groups). *$p < 0.05$, as compared with buffer controls.

was needed because in initial experiments when it was not administered, there was a gradual loss of inhibition and a return of leukocyte rolling. This increase in rolling was rapidly (e.g., 1–2 min) and completely abolished by the second dose of fucoidin (data not shown). The administration of a single higher dose (10 mg/kg) of fucoidin at the beginning of the experiment resulted in a decline in MABP and a resulting decline in RBC velocity and shear rates, so this dose was not used. Experiments in which PB1.3 and LAM1-116 were given simultaneously could also have been utilized to examine the role of E-selectin in this model, but these experiments could not be done due to limited quantities of mAb PB1.3. Data from mAb- and fucoidin-treated animals were compared with buffer control animals and animals given 300 μl of PBS vehicle i.v. 10 min before superfusion of the mesentery with LPS.

Data analysis

All data are presented as mean ± SEM. Data were compared by ANOVA using post hoc analysis with Fischer's corrected *t* test. Probabilities of 0.05 or less were considered statistically significant.

Results

Mesenteric superfusion with LPS increases local leukocyte rolling and adhesion, and decreases leukocyte rolling velocity

Superfusion of a single loop of rat mesentery with LPS resulted in dose- and time-dependent increases in leukocyte rolling and adherence in mesenteric postcapillary venules. All concentrations of LPS utilized (0.1, 0.5, and 1 μg/ml) resulted in significant changes in leukocyte-endothelial interaction when compared with buffer control animals, in which the mesentery was superfused with normal Krebs-Henseleit buffer (Fig. 1, *A*, *B*, and *C*). Superfusion of the rat mesentery with the lowest concentration of LPS (0.1 μg/ml) resulted in a gradual increase in leukocyte rolling that reached

Table I. *Changes in leukocyte rolling and adhesion induced by* P. aeruginosa- *and* S. minnesota-*derived LPS*

Time (min)	Leukocyte Rolling		Leukocyte Adhesion	
	Pseudomonas	*Salmonella*	*Pseudomonas*	*Salmonella*
0	15.2 ± 4.3	13.8 ± 3.2	1.7 ± 0.8	1.6 ± 0.8
30	47.3 ± 12.7	53.5 ± 16.6	2.5 ± 0.9	6.5 ± 3.0
60	130.2 ± 30.2	118.5 ± 5.3	4.2 ± 1.5	7.4 ± 2.5
90	128.2 ± 15.4	123.7 ± 12.8	3.8 ± 2.1	7.5 ± 1.5
120	132.8 ± 20.3	151.8 ± 17.5	5.5 ± 1.3	8.6 ± 2.6

[a] Superfusion of the mesentery with LPS derived from *P. aeruginosa* and *S. minnesota* resulted in similar changes in leukocyte rolling and adhesion as compared with values for *E. coli* (Fig. 1). Data are presented as mean ± SEM for number of rolling (cells/min) or adherent (cells/100 μm of endothelium) cells. $n = 3$ for both groups.

statistical significance by 60 min (Fig. 1*A*). However, this concentration of LPS did not result in any increase in leukocyte adhesion over the 2-h protocol (Fig. 1*B*). The highest concentration of LPS (1 μg/ml) resulted in a more rapid increase in leukocyte rolling and a significant increase in leukocyte adhesion. By 30 min, LPS-induced leukocyte rolling and adhesion were significantly different from buffer controls (rolling, 61.1 ± 4.5 vs 17.4 ± 5.3 cells/min, respectively; adherence, 5 ± 0.6 vs 1.2 ± 0.4 cells/100 μm of venular endothelium, respectively) (Fig. 1, *A* and *B*). Leukocyte rolling was further increased at 60 min (109.6 ± 6.8 cells/min) and 90 min (155.3 ± 27.5 cells/min) and appeared to plateau at this level, as there was no further increase at 120 min (168.3 ± 27.8 cells/min). Using 1 μg/ml, LPS-induced leukocyte adhesion was maximal by 60 min (7 ± 1.6 cells/100 μm of endothelium) and remained at this level to the end of the 2-h superfusion. Although there were similar changes in leukocyte-endothelial interaction observed with 0.5 and 1 μg/ml of LPS, there was greater variation in leukocyte response among animals when 0.5 μg/ml of LPS was utilized and, therefore, 1 μg/ml was utilized for studies examining the role of selectins in LPS-induced leukocyte-endothelial interactions. The changes in leukocyte rolling and adhesion observed with superfusion of the mesentery with 1 μg/ml of LPS derived from *E. coli* were not found to be specific to this bacterial serotype. Superfusion of the mesentery with LPS derived from *P. aeruginosa* or *S. minnesota* resulted in similar changes in leukocyte rolling and adhesion (Table I).

Also of importance in this model were the changes in leukocyte rolling velocity observed with LPS superfusion (Fig. 1*C*). In the microcirculation of buffer control animals, there was no change in leukocyte rolling velocity along the venular endothelium over the 2-h protocol. Superfusion of the mesentery with 1 μg/ml of LPS resulted in a rapid decline in leukocyte rolling velocity that was reduced significantly compared with buffer animals by 30 min (53.2 ± 7.7 vs 85.6 ± 12.3 μm/s, respectively), and remained depressed throughout the observation period.

Superfusion of a single loop of rat mesentery with LPS in Krebs-Henseleit buffer did not result in any significant change in MABP and had no effect on venular diameter, RBC velocity, or venular wall shear rates as compared with buffer control animals (Table II). The lack of significant changes in any of these parameters, even at the 1 μg/ml concentration of LPS, suggests that changes in leukocyte-endothelial interactions observed in this model do not result from hemodynamic alterations.

LPS superfusion of the mesentery has no effect on circulating leukocyte counts or differentials

Introduction of a large quantity of LPS into the circulation has been shown to result in a rapid decline in circulating neutrophil

Table II. *Effects of LPS on mean values for venular diameter, RBC velocity, and venular wall shear rate*[a]

	Time (Min)				
	0	30	60	90	120
Mean venular diameter (μm)					
Buffer control	26.8 ± 1.0	27.3 ± 0.9	27.4 ± 0.9	27.8 ± 1.0	27.5 ± 1.1
0.1 μg/ml LPS	30.2 ± 1.4	30.8 ± 1.5	30.9 ± 1.6	31.0 ± 1.7	31.4 ± 1.7
0.5 μg/ml LPS	28.5 ± 1.7	29.0 ± 1.6	29.3 ± 1.5	29.3 ± 1.5	29.0 ± 1.5
1.0 μg/ml LPS	28.4 ± 1.3	28.8 ± 1.2	29.2 ± 1.1	29.0 ± 1.3	29.0 ± 1.4
Mean RBC velocity (mm/sec)					
Buffer control	1.93 ± 0.20	1.91 ± 0.17	1.78 ± 0.19	1.93 ± 0.17	1.76 ± 0.20
0.1 μg/ml LPS	2.11 ± 0.30	2.05 ± 0.33	1.97 ± 0.27	1.94 ± 0.32	1.88 ± 0.30
0.5 μg/ml LPS	1.89 ± 0.33	1.76 ± 0.26	1.43 ± 0.32	1.84 ± 0.31	1.90 ± 0.30
1.0 μg/ml LPS	1.92 ± 0.16	1.84 ± 0.18	1.90 ± 0.17	1.90 ± 0.21	1.94 ± 0.21
Venular wall shear rate (s^{-1})					
Buffer control	565.9 ± 74.6	559.7 ± 66.3	521.4 ± 65.2	560.5 ± 51.5	514.5 ± 64.8
0.1 μg/ml LPS	534.5 ± 47.8	517.4 ± 60.4	498.3 ± 41.5	487.0 ± 50.8	471.1 ± 46.7
0.5 μg/ml LPS	527.1 ± 105.4	492.7 ± 88.2	486.5 ± 73.5	515.6 ± 98.8	532.8 ± 99.9
1.0 μg/ml LPS	548.8 ± 75.9	529.5 ± 81.8	543.1 ± 76.9	547.3 ± 90.9	556.3 ± 88.9

[a] Mean values for venular diameter, RBC velocity, and venular wall shear rates from buffer control and LPS-treated rats. A single loop of mesentery was superfused with LPS in Krebs-Henseleit buffer at concentrations of 0.1, 0.5, and 1.0 μg/ml. Venular diameter and RBC velocity were measured and venular wall shear rates were quantified at baseline (0 min) and at 30, 60, 90, and 120 min after initiation of LPS superfusion. There was no significant change in venular diameter, RBC velocity, or shear rates with LPS superfusion of the mesentery (n = 6 for all groups).

numbers (8, 45). To determine the effects of mesenteric LPS superfusion on circulating leukocyte numbers, cell counts were measured in arterial samples at 0, 30, 60, 90, and 120 min of LPS superfusion, and leukocyte differentials were determined at 0 and 120 min. The majority of circulating rat leukocytes under baseline conditions were lymphocytes (Fig. 2). Lymphocytes comprised 70 to 80% of circulating leukocytes at 0 min; of the remaining cells, 10 to 20% were neutrophils and 2 to 3% were eosinophils or monocytes. By the end of the 2-h protocol, the ratios had changed reciprocally to 70 to 80% neutrophils and 10 to 20% lymphocytes, even in buffer-superfused controls. Percentages of monocytes and eosinophils remained unchanged. Total circulating leukocyte numbers increased during the 2-h protocol. Numbers of circulating cells were not significantly different among the groups at baseline, and there were similar changes in leukocyte numbers for all groups, including buffer controls (Fig. 3). Because leukocyte differentials and total leukocyte numbers showed similar changes in LPS-treated and control animals, it appears that factors associated with surgical manipulation (e.g., anesthesia, insertion of intravascular lines, exteriorization of the mesentery, etc.), and not mesenteric superfusion with LPS, were responsible for the changes in these leukocyte parameters.

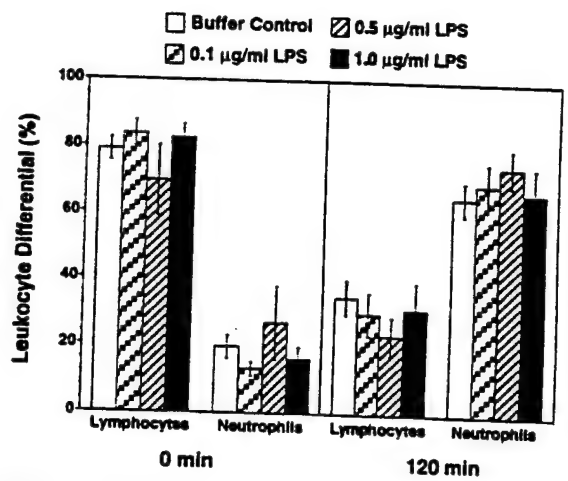

FIGURE 2. Leukocyte differentials from buffer control and LPS-treated rats. Whole blood smears were made at baseline (0 min) and 120 min after LPS superfusion. Bar heights represent mean percentage of neutrophils and lymphocytes (n = 6 for all groups). Percentages of monocytes and eosinophils remained unchanged at 2 to 3%. Increased percentage of neutrophils was seen in all groups, with no significant difference among groups.

Both P- and L-selectin mediate LPS-induced changes in leukocyte rolling

LPS has been demonstrated to induce expression of P-selectin, E-selectin, and ligands for L-selectin on the vascular endothelium in vitro (4, 5, 8). To determine the adhesion mechanism(s) responsible for LPS-induced increases in leukocyte rolling observed in this model, mAb to P-selectin (PB1.3) or L-selectin (LAM1-116) were administered i.v. 10 min before superfusion of the mesentery with 1 μg/ml of LPS. Because mAb that cross-react with rat E-selectin were not available, we utilized fucoidin, a polysaccharide that binds to and blocks the function of P- and L-selectin, but not E-selectin (16, 44), to determine whether there would be any residual rolling or adhesion following blockade of both P- and L-selectin.

Administration of the P-selectin blocking mAb PB1.3 did not alter basal leukocyte rolling, but significantly decreased the number of rolling leukocytes at 30 min of LPS superfusion as compared with vehicle-treated animals (Fig. 4). These findings suggest a rapid, LPS-induced expression of P-selectin, not seen in control animals. By 60 min and out to 120 min, there was a decrease in leukocyte rolling in rats given anti-P-selectin mAb (~20–25%), but these values did not reach statistical significance. Administration of the P-selectin blocking mAb PB1.3 at twice the dose (2 mg/kg) did not result in any further inhibition of leukocyte rolling (n = 3, data not shown). The isotype-matched control mAb, 1E6, given at a dose of 1 mg/kg, had no effect on leukocyte rolling at any of the time points. In contrast to the effects seen with the anti-P-selectin mAb, the L-selectin blocking mAb (LAM1-116) significantly inhibited leukocyte rolling at all time points (~80%) (Fig. 4). The L-selectin-binding, nonblocking control mAb (LAM1-118) did not significantly alter leukocyte rolling at the earlier time points (0 through 60 min), but leukocyte rolling numbers tended to be decreased by 90 min, and this decrease was

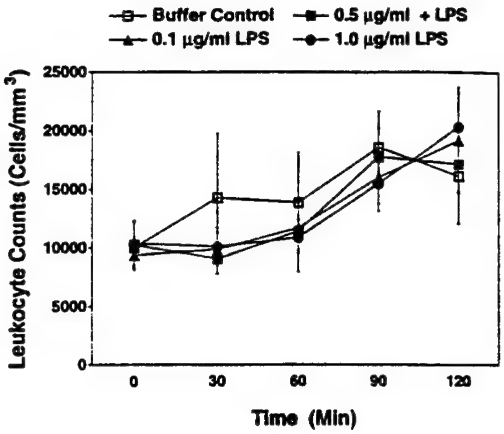

FIGURE 3. Circulating leukocyte counts from buffer control and LPS-treated rats. Arterial blood samples (100 μl) were taken, and total circulating leukocyte numbers were quantified at baseline (0 min) and at 30, 60, 90, and 120 min after initiation of LPS superfusion. Increased total circulating leukocyte numbers were found in all groups, with no significant difference among groups (n = 6 for all groups).

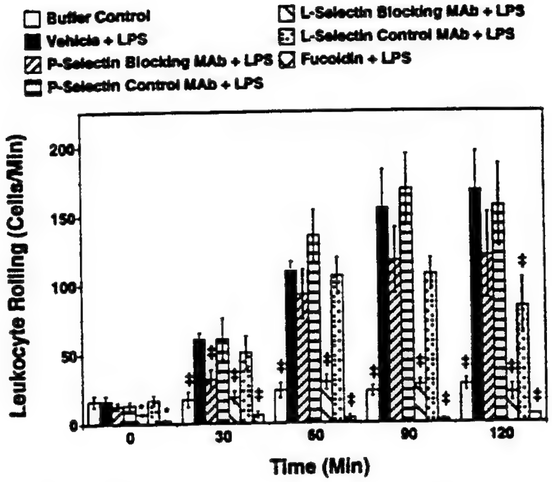

FIGURE 4. P- and L-selectin mediate basal and LPS-induced leukocyte rolling. Values shown represent mean basal and LPS-induced (1 μg/ml) leukocyte rolling in rats pretreated with vehicle (300 μl PBS, n = 6), P-selectin blocking mAb (PB1.3, 1 mg/kg, n = 6), isotype-matched control mAb (1E6, 1 mg/kg, n = 6), L-selectin blocking mAb (LAM1-116, 100 μg/rat, n = 6), L-selectin nonblocking mAb (LAM1-118, 100 μg/rat, n = 6), or fucoidin (5 mg/kg, n = 6), as compared with buffer controls (n = 6). *$p < 0.05$, as compared with buffer control rats at 0 min. ‡$p < 0.05$, as compared with vehicle-treated rats at 30, 60, 90, and 120 min.

significant by 120 min. The decrease in leukocyte rolling with LAM1-118 appeared to be due to the continual decline in circulating cell numbers. Given 10 min before the 0 min or control recording, the L-selectin blocking mAb LAM1-116 was found to significantly inhibit not only LPS-induced increases in rolling, but also basal leukocyte rolling at time 0 (Fig. 4). No such decrease in basal leukocyte rolling was observed with administration of the nonblocking mAb LAM1-118. These data suggest that both P- and L-selectin are necessary for early LPS-induced leukocyte rolling in this model. This conclusion is strengthened by the use of fucoidin that, when given i.v. at a dose of 5 mg/kg 10 min before initiation of LPS and again at 60 min of LPS superfusion, completely inhibited (>98%) basal and LPS-induced leukocyte rolling, confirming that both P- and L-selectin mediate basal and LPS-induced leukocyte rolling in the rat mesentery (Fig. 4). All changes in leukocyte rolling with these antagonists were observed in the absence of changes in venular wall shear rates (Table III). Furthermore, venular diameter and venular wall shear rates were not significantly different among the various treatment groups.

Functional P-selectin, but not L-selectin, is required for LPS-induced leukocyte adhesion

Leukocyte rolling along the vascular endothelium is widely believed to be a precursor to leukocyte adhesion. The selectin family of adhesion molecules is thought to mediate the initial contact between the circulating leukocyte and the vascular endothelium, bringing the leukocyte into contact with tissue or resident cell-derived mediators (e.g., PAF, chemokines, leukotrienes) that stimulate firm adherence and transendothelial migration. We therefore examined our selectin antagonists for their ability to alter cell adhesion, and marked differences were observed. Administration of the P-selectin blocking mAb PB1.3 completely inhibited LPS-induced leukocyte adhesion for the entire 2-h superfusion (Fig. 5). In contrast, the L-selectin mAb LAM1-116, which markedly decreased leukocyte rolling throughout (Fig. 4), had no effect on the number of leukocytes adhering to the endothelium (Fig. 5). Therefore, although L-selectin was observed to mediate the majority (~80%) of leukocyte rolling in rats exposed to LPS, L-selectin rolling was not required for leukocyte adhesion. Neither of the isotype-matched control mAb (i.e., 1E6 and LAM1-118) had any effect on LPS-induced adhesion (Fig. 5). As expected, fucoidin, which blocked both P- and L-selectin rolling, eliminated LPS-induced leukocyte adhesion (Fig. 5). These data suggest that L-selectin, although effective in mediating leukocyte rolling, did not function like P-selectin in initiating the next step of leukocyte-endothelial interaction, leukocyte adhesion, in this model.

Leukocytes roll at different velocities on P- and L-selectin in vivo

As noted above, superfusion of the rat mesentery with LPS resulted in a rapid decline in mean rolling velocity, as compared with non-LPS-treated buffer control animals (53.2 ± 7.7 vs 85.6 ± 12.3 μm/s, respectively, at 30 min), without any significant change in venular wall shear rate. This decrease in leukocyte rolling velocity was attenuated significantly by administration of the anti-P-selectin mAb PB1.3, and in fact, at later time points (i.e., 90 and 120 min), leukocyte rolling velocities in the presence of mAb PB1.3 significantly exceed those seen in buffer control rats (Fig. 6). In LPS-treated animals given the isotype-matched control mAb 1E6, it was observed unexpectedly that mean leukocyte rolling velocity was significantly different from rolling velocity in LPS-treated animals given vehicle at 30 min. However, no significant difference between mAb 1E6 and vehicle-treated animals was noted at later time points, as expected. Regarding the observations at the 30-min time point, leukocyte rolling velocities were found to be very rapid (>100 μm/s) at baseline in two of the rats, and although velocities declined from baseline in these animals, the decline was not sufficient to bring values below those in buffer control animals at 30 min.

Blocking L-selectin-mediated rolling caused a response opposite from that observed with P-selectin blockade. Leukocyte rolling velocities in LAM1-116-treated animals were consistently lower than those seen in vehicle-treated rats, with differences in values reaching statistic significance at later time points (i.e., 90 and 120 min) (Fig. 6). These data confirm previous in vivo (46) findings

Table III. *Comparison of venular diameter and venular wall shear rates for buffer control, vehicle-treated, and mAb-treated rats*[a]

	Venular diameter (μm)	Venular Wall Shear Rate (s^{-1}) 0 min	30 min	60 min	90 min	120 min
Buffer control	27.7 ± 0.90	565.9 ± 74.6	559.7 ± 66.3	521.4 ± 65.2	560.5 ± 51.5	514.5 ± 64.8
Vehicle + LPS	28.8 ± 1.4	548.8 ± 75.9	529.5 ± 81.8	543.1 ± 76.9	547.3 ± 76.9	556.3 ± 88.9
P-selectin blocking mAb + LPS	28.8 ± 0.47	514.8 ± 68.7	485.5 ± 65.5	476.7 ± 64.8	468.5 ± 61.5	519.1 ± 67.2
L-selectin blocking mAb + LPS	29.0 ± 0.86	477.2 ± 55.3	476.6 ± 52.9	479.4 ± 53.0	510.7 ± 47.9	451.1 ± 50.9
Fucoidin + LPS	29.8 ± 1.5	544.8 ± 25.1	471.4 ± 23.6	518.9 ± 47.5	514.1 ± 25.6	441.6 ± 22.2

[a] Mean venular diameter and venular wall shear rates were not significantly different among rats pretreated with vehicle (300 μl PBS, $n = 6$), P-selectin blocking mAb (PB 1.3, 1 mg/kg, $n = 6$), L-selectin blocking mAb (LAM1-116, 100 μg/rat, $n = 6$), or fucoidin (5 mg/kg, $n = 6$), as compared with buffer controls ($n = 6$).

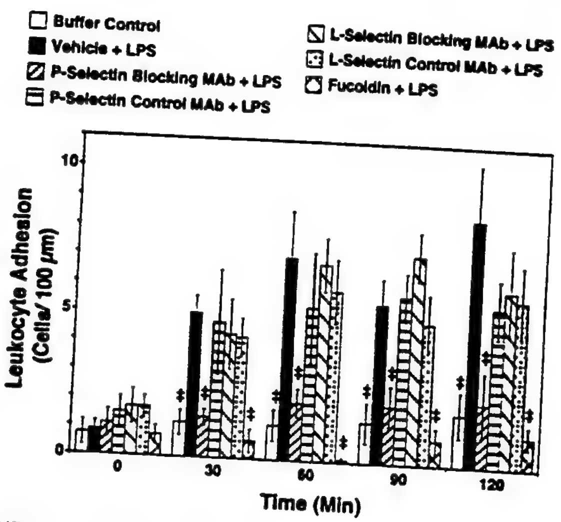

FIGURE 5. P-selectin, but not L-selectin, is necessary for LPS-induced leukocyte adhesion. Values shown represent mean basal and LPS-induced (1 μg/ml) leukocyte adhesion in rats pretreated with vehicle (300 μl PBS, $n = 6$), P-selectin blocking mAb (PB1.3, 1 mg/kg, $n = 6$), isotype-matched control mAb (1E6, 1 mg/kg, $n = 6$), L-selectin blocking mAb (LAM1-116, 100 μg/rat, $n = 6$), L-selectin nonblocking mAb (LAM1-118, 100 μg/rat, $n = 6$), or fucoidin (5 mg/kg, $n = 6$), as compared with buffer controls ($n = 6$). ‡$p < 0.05$, as compared with vehicle-treated rats.

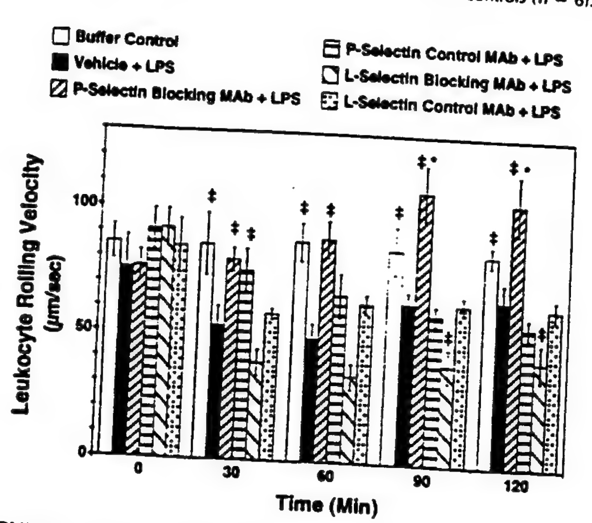

FIGURE 6. Leukocytes roll at different velocities on P- and L-selectin in vivo. Values represent mean basal and LPS-induced (1 μg/ml) changes in leukocyte rolling velocity in rats pretreated with vehicle (300 μl PBS, $n = 6$), P-selectin blocking mAb (PB1.3, 1 mg/kg, $n = 6$), isotype-matched control mAb (1E6, 1 mg/kg, $n = 6$), L-selectin blocking mAb (LAM1-116, 100 μg/rat, $n = 6$), L-selectin nonblocking mAb (LAM1-118, 100 μg/rat, $n = 6$), or fucoidin (5 mg/kg, $n = 6$), as compared with buffer controls ($n = 6$). *$p < 0.05$, as compared with buffer control rats. ‡$p < 0.05$, as compared with vehicle-treated rats.

and strongly suggest that, under similar shear conditions, leukocytes roll at very different velocities on P- and L-selectin in vivo.

Effects of mAb and fucoidin on total circulating leukocyte counts and differentials

Infusion of either the blocking (LAM1-116) or nonblocking (LAM1-118) L-selectin mAb resulted in a rapid (i.e., within 10 min) and sustained decrease in circulating leukocyte counts (Fig. 7). Before administration of LAM1-116, rats had a mean circulating leukocyte count of 10,200 ± 1,278 cells/mm^3. Ten minutes after mAb administration, mean leukocyte counts were 6,350 ± 1,728 cells/mm^3. A similar decline was seen with the nonblocking LAM1-118 mAb (i.e., 10,333 ± 1,434 vs 7,750 ± 898 cells/mm^3). These findings were consistent with other studies in which L-selectin-binding mAb have been utilized. The effect appears to be due to transient sequestration of leukocytes in the lung and liver following binding of the mAb to L-selectin (Tedder, T. F., et al., unpublished observation). Changes in circulating leukocyte numbers were consistent with the observation that the number of rolling leukocytes in rats treated with the nonblocking mAb LAM1-118 was depressed at 120 min when compared with vehicle-treated rats (Fig. 4). However, as numbers of rolling and adherent leukocytes in LAM1-118-treated animals were not significantly different from vehicle-treated animals for the majority of the protocol, results obtained with the L-selectin blocking mAb LAM1-116 were not simply due to decreased numbers of circulating cells. In support of this conclusion, infusion of fucoidin, which completely blocked leukocyte rolling, resulted in elevated basal leukocyte counts ($p < 0.05$, as compared with buffer control), with no effect on leukocyte counts at later time points. Of interest, the change in leukocyte differentials observed in all other treatment groups (i.e., shift from greater lymphocyte numbers to greater neutrophil numbers) was reduced in fucoidin-treated animals (Fig. 8).

Neither PB1.3 nor 1E6 had any effect on the number of circulating leukocytes at any of the time points (Fig. 7). In the case of PB1.3 infusion, there was a trend toward increased circulating leukocyte numbers in the later stages of the experiments, but this did not reach statistical significance. Similarly, PB1.3 did not affect leukocyte differentials at 0 and 120 min (Fig. 8).

Discussion

The sequelae of pathologic events associated with systemic infection with Gram-negative bacteria are mediated predominantly by LPS (2). Although LPS acts on a variety of cell types, many of the

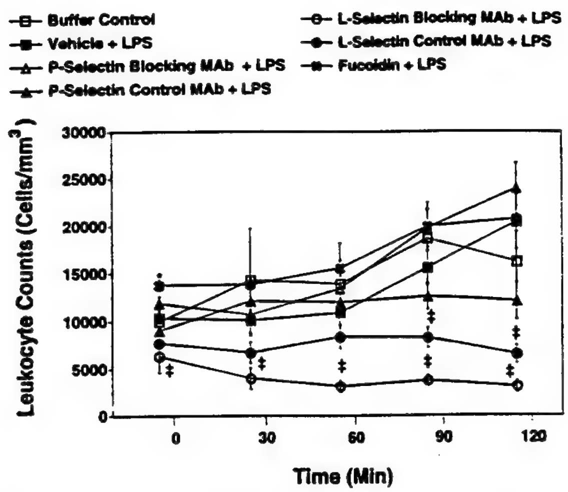

FIGURE 7. Effects of mAb and fucoidin pretreatment on circulating leukocyte counts. Values represent mean circulating leukocyte counts in rats pretreated with vehicle (300 μl PBS, $n = 6$), P-selectin blocking mAb (PB1.3, 1 mg/kg, $n = 6$), isotype-matched control mAb (1E6, 1 mg/kg, $n = 6$), L-selectin blocking mAb (LAM1-116, 100 μg/rat, $n = 6$), L-selectin nonblocking mAb (LAM1-118, 100 μg/rat, $n = 6$), or fucoidin (5 mg/kg, $n = 6$), as compared with buffer controls ($n = 6$). *$p < 0.05$, as compared with buffer control rats. ‡$p < 0.05$, as compared with vehicle-treated rats.

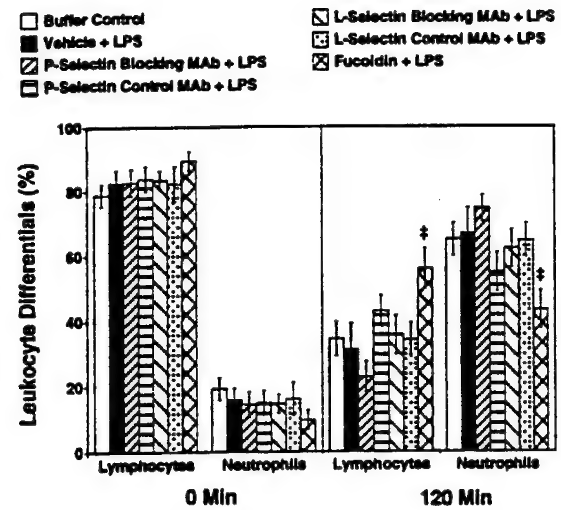

FIGURE 8. Effects of mAb and fucoidin pretreatment on leukocyte differentials. Values represent mean leukocyte differentials in rats pretreated with vehicle (300 μl PBS, $n = 6$), P-selectin blocking mAb (PB1.3, 1 mg/kg, $n = 6$), isotype-matched control mAb (1E6, 1 mg/kg, $n = 6$), L-selectin blocking mAb (LAM1-116, 100 μg/rat, $n = 6$), L-selectin nonblocking mAb (LAM1-118, 100 μg/rat, $n = 6$), or fucoidin (5 mg/kg, $n = 6$), as compared with buffer controls ($n = 6$). ‡$p < 0.05$, as compared with vehicle-treated rats.

most detrimental effects of LPS are thought to be mediated through its effects on the vascular endothelium. Acting both directly and indirectly on the endothelial cell, LPS can increase gene expression of numerous proteins associated with the inflammatory response. For instance, LPS directly increases the expression of the inducible nitric oxide synthase, and thus nitric oxide, which is believed to contribute to the hypotension associated with endotoxic shock (47). Furthermore, through direct stimulation of endothelial cell cytokine production (i.e., IL-1, TNF-α) (9, 11, 12, 31) and endothelial cell adhesion molecule expression (i.e., selectins, ICAM-1) (4, 5, 8, 26), and indirectly through stimulation of circulating leukocytes (18) and tissue resident cells (37), LPS enhances leukocyte infiltration into tissues. Excessive leukocyte recruitment into tissues leads to syndromes, such as adult respiratory distress syndrome, which contribute to endotoxic shock-induced mortality.

Despite the well-recognized consequences of endotoxemia, the adhesion-related mechanisms responsible for cell recruitment events in vivo remain poorly characterized. In the current study, we have developed a model of rat intravital microscopy to investigate LPS-induced leukocyte recruitment, with an initial focus on the role of the selectin family of adhesion molecules in LPS-induced changes in leukocyte-endothelial interactions. Superfusion of the rat mesentery, with concentrations of LPS that did not alter venular wall shear rates, resulted in a rapid increase in leukocyte rolling and adhesion. Decreases in venular wall shear rate are of concern because decreased shear rates, in the absence of tissue stimulation, can result in increased leukocyte rolling and adhesion (48). The time course and magnitude of LPS-induced changes in leukocyte rolling and adhesion, as well as changes in leukocyte rolling velocity, were dependent on LPS concentration. Low concentrations of LPS (0.1 μg/ml) resulted in a gradual increase in leukocyte rolling that was not accompanied by increased leukocyte adhesion or decreased leukocyte rolling velocity, while higher concentrations of LPS (1 μg/ml) resulted in rapid increases in both rolling and adhesion and a rapid decline in leukocyte rolling velocity. These data support in vitro findings that demonstrate that LPS, even at very low concentrations (0.1–1 μg/ml), can up-regulate endothelial adhesion molecules and their ligands on HUVEC (4, 5, 20). Higher concentrations of LPS were not utilized in these studies, as previous in vivo data demonstrate that higher concentrations of LPS (i.e., 100 μg/ml) can decrease venular wall shear rates (37). Changes in leukocyte rolling and adhesion observed with LPS superfusion were not specific to the *E. coli* source of the LPS, as similar results were seen utilizing LPS derived from two other bacterial sources (Table I). These results are not surprising, as the most biologically active component of LPS, lipid A, is highly conserved among Gram-negative bacteria (2).

In the present system, we found both P- and L-selectin to be important in basal and LPS-induced leukocyte-endothelial interactions, although the role of each of the molecules was different. Most of the basal or spontaneous leukocyte rolling in this system appeared to be L-selectin mediated, with less contribution from P-selectin. As previously reported, administration of the P-selectin neutralizing mAb, PB1.3, did not significantly alter basal leukocyte rolling or adhesion (42), while administration of an L-selectin mAb significantly attenuated, but did not eliminate, basal leukocyte rolling (49). Although changes in basal leukocyte rolling were not altered significantly by PB1.3, data from experiments utilizing fucoidin suggest some function for P-selectin in baseline rolling, as fucoidin further decreased leukocyte rolling below numbers seen with LAM1-116 (Fig. 4). Thus, it appears that L-selectin was primarily mediating leukocyte rolling at baseline, with some contribution of P-selectin.

Both P- and L-selectin mediated LPS-induced leukocyte rolling at 30 min, the earliest time point examined. Contrary to its effects on basal rolling, administration of mAb PB1.3 significantly decreased leukocyte rolling during the first 30 min of superfusion with LPS, indicating an LPS-induced up-regulation of P-selectin. Likewise, the anti-L-selectin mAb also inhibited early leukocyte rolling. Administration of fucoidin completely eliminated rolling

at 30 min, confirming both P- and L-selectin-mediated rolling at this time point.

The ability of LPS to rapidly up-regulate P-selectin in vivo has been previously demonstrated. Coughlan et al. (8) showed that the rapid (i.e., 5–10 min) leukopenia and tissue sequestration of neutrophils associated with i.v. infusion of LPS in the rat were inhibited by prior administration of an anti-P-selectin mAb. These authors (8) also reported that in vitro stimulation of cultured HUVEC with LPS (1 μg/ml) resulted in a rapid increase in surface expression of P-selectin. However, Khew-Goodall et al. (50) found no effect of LPS on HUVEC levels of P-selectin mRNA or surface expression of P-selectin, and we were unable to demonstrate a significant increase in P-selectin-mediated neutrophil adhesion to primary cultures of HUVEC stimulated with LPS for 10 to 30 min (unpublished observations). Thus, further studies are required to determine whether in vivo up-regulation of P-selectin is a direct or indirect effect of LPS on the endothelium.

Although the anti-P-selectin mAb inhibited approximately 50% of the early increases in leukocyte rolling (i.e., 30 min), this mAb was less effective at inhibiting leukocyte rolling at later time points (i.e, ~20–25% inhibition). The L-selectin blocking mAb LAM1-116, on the other hand, significantly inhibited leukocyte rolling at all time points. As direct stimulation of leukocytes with LPS has been demonstrated to result in L-selectin shedding (18), the finding that LPS increased L-selectin rolling implies increased endothelial expression of an L-selectin ligand, and also implies that superfusion of the mesentery with LPS had greater activating effects on the local vascular endothelium than on circulating leukocytes. Although LPS has been demonstrated to increase the expression of an L-selectin ligand on endothelium (5), the ligand remains uncharacterized and the time course of its induction is incompletely defined. For instance, in vitro stimulation of HUVEC with LPS for at least 2 h increases expression of an L-selectin ligand, but earlier time points have not been examined (5). The current study indicates that an L-selectin ligand is rapidly up-regulated in vivo in sufficient quantity to mediate leukocyte rolling, even under normal shear conditions.

Although the anti-L-selectin mAb reduced the number of rolling cells to values similar to those in buffer control animals, administration of fucoidin further decreased leukocyte rolling. These data imply that not all of the leukocyte rolling at later time points was L-selectin mediated, and that P-selectin is still involved, although to a lesser degree. A continued role for P-selectin at later time points is clearly supported by leukocyte adhesion data. Although L-selectin mediated a greater portion of LPS-induced rolling between 60 and 120 min, blocking L-selectin-mediated rolling did not inhibit leukocyte adhesion. However, the anti-P-selectin mAb, which blocked only a small portion of later leukocyte rolling, completely inhibited leukocyte adhesion. Thus, the small number of leukocytes observed to be rolling on P-selectin following L-selectin blockade was sufficient to facilitate significant leukocyte adhesion. This enhanced role for P-selectin in leukocyte adhesion is supported by studies of Gaboury et al. (51), who utilized a model of rat intravital microscopy similar to the one used in this study, to demonstrate that blockade of P-selectin with mAb PB1.3 effectively inhibited the leukocyte rolling and adhesion induced by the mast cell degranulating agent, compound 48/80. These data indicate a requirement for P-selectin in both rolling and adhesion in vivo. In this study (51), the ability of mAb PB1.3 to block adhesion was thought to be due to a decrease in total leukocyte rolling (i.e., decreased P-selectin-mediated rolling decreases total cell interaction with the endothelium and, therefore, adhesion). However, the data presented in this study indicate that P-selectin may play a different role in leukocyte adhesion than previously thought. We show that selective blockade of P-selectin-mediated rolling, without blockade of other selectin-mediated rolling, was sufficient to abolish leukocyte adhesion. One explanation for these findings may be that neutrophil activation for firm adhesion by chemotactic factors can be enhanced by binding to P-selectin, a phenomenon not documented with L-selectin binding. Lorant et al. (52) demonstrated that intracellular Ca^{2+} elevations, β_2 integrin expression, and cellular shape changes were facilitated in neutrophils that adhered to endothelial cells expressing P-selectin and the chemotactic agent, PAF. Although binding of P-selectin alone does not activate the leukocyte, it appears that P-selectin-mediated leukocyte binding is effective in bringing unstimulated leukocytes in contact with endothelial-expressed mediators, such as PAF. Whether PAF plays a role in LPS-induced leukocyte-endothelial interactions in this model remains to be determined.

Some insight into the differences in the abilities of P- and L-selectin to mediate leukocyte adhesion may also be gained from data concerning leukocyte rolling velocity. As noted in vitro, it appears that the strength of the interaction between the selectin molecules and their ligands varies among the selectins, and may dictate the speed at which leukocytes roll (53, 54). In the present study, LPS-induced, L-selectin-mediated leukocyte rolling velocity (e.g., rolling velocity in the presence of the P-selectin neutralizing mAb PB1.3) was very rapid, while P-selectin-mediated leukocyte rolling velocity (i.e., rolling velocity in the presence of the L-selectin neutralizing mAb LAM1-116) was very slow (Fig. 6). These data are consistent with findings from Jung et al. (46), who demonstrated slower rolling velocities on P-selectin than L-selectin in a murine model of intravital microscopy. As P-selectin mediates slower rolling, it may be more efficient than L-selectin in bringing leukocytes in contact with chemotactic agents on the endothelial surface capable of up-regulating β_2 integrins and facilitating leukocyte adhesion and transendothelial migration.

The lack of L-selectin-dependent leukocyte adhesion in this model system is surprising, as a role for L-selectin in inflammation-induced leukocyte recruitment has been demonstrated clearly in other model systems, such as the L-selectin-deficient mouse (38). The belief that the selectins act in concert with other mediators (i.e., chemoattractants, cytokines, chemokines) to facilitate firm adhesion may offer insight into our findings. The duration of our model may not be sufficient to allow maximal expression of cytokines such as IL-1 and TNF-α, which may be necessary to facilitate L-selectin-induced adhesion. Although the explanation of these results is not clear at this time, the findings do provide novel insight into selectin function, as it appears that the selectins can mediate leukocyte rolling that does not result in adhesion.

As noted, treatment of animals with fucoidin also inhibited leukocyte adhesion in this system. These findings are contrary to in vitro studies, which have reported that fucoidin does not inhibit leukocyte adhesion (16, 44). A potential explanation for these findings lies within differences in leukocyte adhesion under flow conditions. Kubes et al. (44) recently reported that fucoidin, administered in a model of cat mesenteric ischemia/reperfusion, only inhibited leukocyte adhesion in animals in which RBC velocity in the vessel after reperfusion was >70% of preischemic values. Thus, fucoidin-mediated blockade of leukocyte adhesion was dependent upon venular wall shear rate. Our data support these findings, as fucoidin was extremely effective in inhibiting leukocyte adhesion when shear rates did not decline. These findings may also, in part, give another insight into the lack of L-selectin-mediated leukocyte adhesion. In vitro data suggest that L-selectin-mediated leukocyte arrest (i.e., adhesion) increases with decreasing shear force, thus implying that decreased venular wall shear

rates may be required for L-selectin to better facilitate leukocyte adhesion (5).

Although our data do not suggest a role for E-selectin in this model of LPS-induced leukocyte-endothelial activation, we cannot definitively rule out E-selectin participation because of the potential interaction of L-selectin with E-selectin and P-selectin (55, 56). Picker et al. (56) demonstrated a role for L-selectin in presentation of carbohydrate ligands to E- and P-selectin, and were able to inhibit neutrophil adhesion to E-selectin-transfected cells with an anti-L-selectin mAb. If L-selectin interacted with endothelial E-selectin in our system, use of LAM1-116 and fucoidin may mask our ability to distinguish E-selectin-mediated rolling. Although E-selectin may interact with L-selectin in this model, it is unlikely, as Spertini et al. (5) demonstrated that L-selectin-mediated leukocyte adhesion to LPS-stimulated HUVEC was not mediated via E-selectin, but instead through the up-regulation of a separate L-selectin ligand. A more likely explanation for the lack of E-selectin-mediated rolling in our model is the short duration of superfusion and observation utilized herein. Although LPS at concentrations as low as 100 ng has been demonstrated to increase E-selectin mRNA in HUVEC in as rapidly as 1 h, levels are not maximum until 4 h (4, 25). If expression follows a similar pattern, our protocol may have missed maximum E-selectin expression. Thus, there may have been some E-selectin expressed on the vascular endothelium at 1 to 2 h, but it may not have been present in sufficient quantity to mediate leukocyte rolling under normal shear conditions. In the present study, the protocol was not extended to further investigate a role for E-selectin because spontaneous leukocyte rolling and adhesion in buffer control animals began to increase beyond 2.5 h.

In conclusion, our data suggest that LPS superfusion of the rat mesentery results in P- and L-selectin-mediated increases in leukocyte rolling and adhesion. Although the roles of each molecule appear to overlap, each molecule mediates a distinct function. P-selectin mediates a smaller portion of leukocyte rolling, but is more effective in facilitating leukocyte adhesion under normal shear conditions. L-selectin, on the other hand, mediates a greater portion of leukocyte rolling, but is less effective in facilitating leukocyte adhesion. Thus, P- and L-selectin play important, but distinct roles in LPS-induced leukocyte-endothelial interaction.

Acknowledgments

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1573 VLA-4 expression on memory/activated CD4+ T cells and their adhesion are upregulated by antigen stimulation. *M. Tarkowski, K. Pacheco, L.J. Rosenwasser,* National Jewish Center for Immunology and Respiratory Medicine, Denver, CO

VLA-4 is expressed on T lymphocytes, and after stimulation it acutely increases its binding avidity. We have shown that allergen stimulation increases VLA-4 receptor density on human CD3+ T cells over 24 to 48 hrs. We hypothesized that the rise was specific for CD4+ cells and correlated with increased cell binding to the counter ligand. Human T cell lines were established after 2 cycles of stimulation with Lol p I allergen (10 mg/ml) or Tetanus toxoid (.2 lfu/ml). Cells were analyzed by flow cytometry at 0, 24, and 48 hrs for staining with antibodies against CD49d, CD4, CD45RO and CD45RA. Parallel T cell samples were labelled with Cr^{51} and incubated for 1 hr in wells coated with the CS-1 fragment of fibronectin or whole plasma fibronectin. Messengar RNA for expression of a-4, b-1 and b-7 chains of VLA-4 was analyzed by RT-PCR. VLA-4 receptor density increased by 85% ($p < 0.05$) 24 hrs after antigen stimulation, exclusively on CD45RO+/CD4+ cells. Binding to CS-1 was coordinately upregulated from 4.5% at baseline to 21% 24 hrs after stimulation. The increased surface expression of VLA-4 correlated with increased a-4 and b-1 chain mRNA expression, but not with b-7 mRNA. Increases were seen with both Lol p I and Tetanus stimulation. These findings indicate that allergen and antigen stimulation induces increased VLA-4 expression on CD45RO+/CD4+ T cells and functionally correlates with enhanced binding to CS-1. We postulate this may be one of the mechanisms to localize allergen specific CD45RO+/CD4+ cells to sites of allergic inflammation.

1574 Regulation of ICAM-1 and VCAM-1 Expression in the Human Bronchial Epithelial Cell Line BEAS-2B and Involvement in Eosinophil Adhesion. *J Atsuta, SA Sterbinsky, LM Schwiebert, BS Bochner, RP Schleimer,* Johns Hopkins Asthma and Allergy Center, Baltimore, MD

We have demonstrated previously (JACI 292:97) that cytokines induce surface expression of ICAM-1 and VCAM-1 on a human bronchial epithelial cell line (BEAS-2B) in vitro. We have now studied 1) mRNA expression of ICAM-1 and VCAM-1 induced by cytokines, 2) relevance of ICAM-1 and VCAM-1 expression on BEAS-2B to eosinophil (EOS) adhesion, and 3) the effect of glucocorticoid. Using Northern blot analysis, ICAM-1 and VCAM-1 mRNA expression was detected in BEAS-2B cells stimulated with TNFα (1 ng/ml, 2 hr). Treatment of BEAS-2B monolayers with TNFα (10 ng/ml, 24 hr) significantly increased adhesion of EOS (from 5.7±0.3% to 15.7%±3.0 adhesion, $p<0.01$). Blocking antibody to ICAM-1 had no significant effect on levels of EOS adhesion. In contrast, antibody to VCAM-1 completely decreased net EOS adhesion (104.3±0.1% inhibition, $p<0.01$). Glucocorticoid (10^{-7} M) had no significant effect on TNFα-induced expression of either ICAM-1 protein or mRNA but significantly inhibited both TNFα-induced VCAM-1 protein and mRNA expression. These results suggest that VCAM-1 on airway epithelium may functionally interact with EOS and that suppression of epithelial VCAM-1 expression by glucocorticoids may contribute to their antiinflammatory effects.

1575 Intercellular Adhesion Molecule-1(CD54) on Eosinophils Is Involved in Cytokine-Stimulated Eosinophil Degranulation. *S Horie, Y Okubo, M Hossain, T Momose, M Sekiguchi,* Shinshu University, Matsumoto city, Japan

Recent evidence suggests that adhesion molecules play important roles in eosinophil functions such as degranulation and superoxide anion production. CD11b/CD18 (Mac-1) and CD49d/CD29 (VLA-4) are involved in eosinophil-endothelial adhesion through their counter ligands, intercellular adhesion molecule-1 (CD54) and vascular cell adhesion molecule-1, respectively. CD54 is also induced on eosinophils by cytokine stimulation. We hypothesized that CD54 on human eosinophils may participate in eosinophil degranulation. Eosinophils were isolated from peripheral blood of normal volunteers by using magnetic cell separation system. CD54 was induced on purified eosinophils by a combination of 10 ng/ml GM-CSF and 10 ng/ml TNF-α within 2 hours of incubation as determined by flow cytometric analysis. GM-CSF alone also induced slight but significant CD54 expression on eosinophils. Eosinophil degranulation was induced by 10 ng/ml GM-CSF on 96-well tissue culture plate coated with human serum albumin and this effect was synergistically enhanced by adding 10 ng/ml TNF-α. To determine the role of newly expressed CD54 in eosinophil degranulation, a blocking assay was performed using monoclonal Abs (mAb) against CD54 and CD18. Anti-CD18 mAb and anti-CD54 mAb markedly inhibited eosinophil degranulation induced by GM-CSF or a combination of GM-CSF and TNF-α (GM-CSF/TNF-α). On the other hand, anti-CD54 mAb had little effect on eosinophil adhesion induced by GM-CSF or GM-CSF/TNF-α, whereas anti-CD18 mAb significantly inhibited eosinophil adhesion. These results indicate that CD54 on eosinophils plays an important role in the eosinophil degranulation by interacting with β2 integrins expressed on eosinophils.

1576 Expression of a novel β2 integrin (αdβ2) on human leukocytes and mast cells. *MH Grayson, M Van der Vieren,* WM Gallatin,* PA Hoffman,* BS Bochner,* Baltimore, MD and *Bothell, WA

β2 integrins are involved in leukocyte adhesion and migration. Recently, a fourth member of the β2 integrin subfamily, αd, was identified. We studied the relative distribution of αd on human leukocyte subtypes and skin mast cells. Partially purified leukocytes or dispersed skin mast cells were analyzed by dual color cytometry for expression of β2 integrin subunits using the following murine mAbs: CD11a (MHM24), CD11b (H5A4), CD11c (BU-15), and αd (169A) (a non-binding IgG1 mAb was used as a control). αd was expressed on all peripheral blood leukocytes but not on skin mast cells. Overall, monocytes expressed the highest density of αd (10.7 ± 1.8 fold IgG control; $\bar{x}$ ± SEM, n = 9) followed by a 30% subpopulation of CD8+ lymphocytes (9.5 ± 3.4, n = 8), basophils (8.2 ± 1.8, n = 7), CD16+ lymphocytes (6.5 ± 2.8, n = 8), neutrophils (6.1 ± 0.8, n = 7), CD19+ lymphocytes (6.1 ± 3.2, n = 8), CD4+ lymphocytes (3.4 ± 1.3, n = 8), and eosinophils (3.0 ± 0.6, n = 11). For most cells, levels of CD11a and CD11b were at least 4 times the levels of αd. Levels of CD11c and αd were similar except for monocytes and neutrophils where CD11c was present at twice the density. Eosinophils appear to have preformed stores of both CD11b and αd, because incubation with phorbol ester (10 ng/ml, 15 min, 37° C) caused a 3 fold increase in expression of αd and a 2 fold increase in CD11b. We conclude that αd is expressed, albeit at different levels, on most circulating leukocytes and, in eosinophils, can be acutely upregulated with phorbol ester. This differs from αd distribution in tissues, where its expression occurs in a more restricted pattern on subsets of leukocytes. The role of αdβ2 integrins in leukocyte adhesion and migration remains to be determined.

Gender	Age	Duration of drug	Clinical presentation
male	77	pravastatin 3yrs	left ventricular dysfunction, polymyalgia, ESR 66
male	66	lovastatin 4yrs	dyspnea, polymyalgia syndrome, ESR 32, ANA 1:160
female	76	lovastatin 1yr	fibrositis, polymyalgia, dyspnea
female	80	simvastatin 3yrs	dyspnea, ESR 89
male	49	pravastatin 4yrs	urticaria, angioedema, ANA 1:80
male	54	lovastatin 9yrs	urticaria, angioedema, ANA 1:320
male	53	pravastatin .5yrs	angioedema, dyspnea
female	69	pravastatin 6yrs	dyspnea, hypersensitivity alveolitis
female	77	pravastatin 3yrs	urticaria
male	73	pravastatin 3yrs	urticaria, rash, ANA 1:80

One patient presented with cough and dyspnea. The high resolution chest tomography (HRCT) showed evidence of alveolitis. The open lung biopsy showed patchy bronchiolitis and alveolitis with collections of histiocytes suggestive of granuloma formation, consistent with hypersensitivity pneumonitis. Subsequent HRCT showed resolution of the alveolitis after stopping the drug. This hypersensitivity syndrome to the lipid lowering statin drugs remains rare. The reported incidence is low at less than 1%. We are including yet another rare but potentially life-threatening complication of hypersensitivity pneumonitis.

219 Incidence and Evaluation of Local Anesthetic Drug Reactions Over a Ten Year Period *TL Heinly, P Lieberman, MS Blaiss,* University of TN, Memphis, TN

Local anesthetic drug reactions are a major source of allergy referrals. In this study we reviewed the records of 494 patients referred for evaluation of drug reactions over the past ten years in a university affiliated private practice. Local anesthetic reactions accounted for 30% of the referrals for drug reactions (149 of 494). Patients ranged in age from eleven to seventy-nine years. Females represented 75% of the population studied, 111 of 149. Reasons for referral included anticipated dental work, minor surgical procedures, and cardiac catheterization. Reported reactions included shortness of breath (22%), mucosal swelling (22%), rash (17%), palpitations (13%), near syncope (13%), nausea (10%), and loss of consciousness (8%). Because of severe drug reactions, emergency room treatment was required in 13 of 149 patients - a substantial nine percent.

All 149 patients underwent skin prick and intradermal testing followed by graded challenge via subcutaneous injection. Of the 149 patients who underwent testing two exhibited a questionably positive response. Both patients underwent subsequent uneventful challenge to a related local anesthetic as shown below..

Patient	Presenting Symptom	Anesthetic	Skin Prick	Intradermal	Challenge
1	Syncope,	mepivacaine	1+	—	—
	hypotension	lidocaine	neg	neg	neg
2	Mucosal	mepivacaine	neg	1+	—
	swelling	lidocaine	neg	neg	neg

These observations are in keeping with previous studies indicating that the vast majority of reactions to local anesthetics are not IgE mediated, and patients can be allowed to receive these agents through the utilization of the graded challenge procedure.

220 αdß2 integrin is an alternative ligand for VCAM-1. *MH Grayson, M Van der Vieren*, WM Gallatin*, PA Hoffman*, BS Bochner,* Johns Hopkins Univ., Baltimore, MD and *ICOS Corp., Bothell, WA.

Two integrins, α4ß1 and 4ß7, have been shown to bind to VCAM-1. We report that the most recently described ß2 integrin, αdß2, a ligand for ICAM-3, also functions as a ligand for VCAM-1. Chinese hamster ovary (CHO) cells were transfected with human αd and ß2 (αdCHO) and were used in flow cytometric assays and in adhesion assays employing immobilized recombinant adhesion molecules. By flow cytometry, the αdCHO cells expressed αd and ß2, but none of the other ß2 integrin α chains or α4; the parental CHO cells (pCHO) did not express α4 or any ß2 integrins. αdCHO cells bound to VCAM-1 (14.2±3.6%; mean adhesion±sem, n=7). VCAM-1 binding was completely blocked using an mAb against the first domain of VCAM-1 (3.0±0.4%; n=3). This was lower than adhesion to BSA (7.5±3.7%; n=7), but was similar to αdCHO binding to E-selectin (2.8±1.5%; n=2). pCHO failed to adhere to VCAM-1, E-selectin, or BSA (<2% adhesion; n=2-4). αd levels on the αdCHO cells slowly declined with serial passage; adhesion to VCAM-1 also declined in parallel. We next hypothesized that leukocytes with elevated αdß2 levels will use this integrin to bind to VCAM-1. Peripheral blood eosinophils were cultured for 5-7d in 10ng/ml IL-5. This increased levels of αd by 2-4 fold, while α4 levels remained unchanged. Adhesion cultured eosinophils to VCAM-1 was 28.8±11.6% (n=3), and was partially but equally inhibited by an mAb against ß2 (17.1±5.0%, n=5) or α4 (18.1±3.4%, n=4). These data suggest that αdß2 is a ligand for VCAM-1. Additional studies with αd blocking mAb are needed to elucidate the relative importance and affinity of αd versus α4 in binding to VCAM-1.

221 Mast Cell IL-4 Release is Related to the Initial Expression of VCAM-1 and the Development of Pulmonary Eosinophilia in a Mouse Asthma Model. *DT Brody, D Kojima, and DD Metcalfe,* Laboratory of Allergic Diseases, NIAID, Bethesda, MD.

While activated mast cells are known to release and generate mediators after activation that are involved in the immediate allergic response, the relevance of mast cell cytokine production is less well understood. In order to investigate the relevance of mast cell cytokine production, we hypothesized that there is a relationship between mast cell cytokine production and subsequent inflammatory events. To explore this issue we first examined the temporal sequence of cytokine production in a mouse model of asthma. The first cytokine mRNAs to be expressed in the lung after antigen challenge were IL-4 and TNF-α, appearing 100 min after challenge. RNAs for IL-5 and IFN-γ were detected at 6 hrs, and remained elevated at 24, 48 and 72 hrs after antigen challenge. As IL-4 is known to be an important cytokine in allergic disease, and since IL-4 deficient mice develop markedly attenuated allergic pulmonary inflammation, we decided to focus on the role of early IL-4. In situ hybridization followed by staining with toluidine blue showed that mast cells located within the alveolar septa were the primary source of early IL-4. Administration of a single neutralizing dose of anti-IL-4 antibody immediately prior to antigen challenge resulted in the disappearance of IL-5 mRNA from the 6 and 24 hr time points, and a corresponding reduction in the number of BAL eosinophils and pulmonary tissue eosinophilia. This effect was transient, as both eosinophils and IL-5 mRNA reappeared at later time points. The fall in IL-5 mRNA correlated directly with a fall in the mRNA for VCAM-1. These findings suggest that in this model, mast cell IL-4 production plays an important role in the initiation of subsequent inflammatory events.

ADHESION MOLECULES *in* ALLERGIC DISEASE

edited by

BRUCE S. BOCHNER
Johns Hopkins Asthma and Allergy Center
Johns Hopkins University School of Medicine
Baltimore, Maryland

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Preface

Over the past few decades, our understanding of the pathophysiology of allergic diseases, including bronchial asthma, allergic rhinitis, and atopic dermatitis, has evolved from a primary focus on IgE, mast cells, and their roles in initiating allergic reactions, to the study of more downstream events such as late-phase responses and the inflammation that accompanies clinically significant chronic allergic diseases. We now realize that preferential migration of human eosinophils, basophils and T lymphocytes, especially those of the Th2 subtype, occurs during allergic inflammatory responses in the skin and airways. Further, these cells and their products play a critical role in producing allergic inflammation, and allergic diseases are the net result of IgE-dependent inflammatory cascades involving certain cells and mediators. These discoveries have fueled efforts to understand the mechanisms involved in selective recruitment processes that differ in other forms of inflammation.

Inflammation has classically been viewed as an interplay between cellular and fluid elements in blood with like constituents in tissues. Under this paradigm, local endothelium and epithelium were thought to play relatively inactive roles, functioning exclusively as barriers. However, it has been known for more than a century that structural changes in these cells can occur at sites of inflammatory reactions, in association with the acquisition of an ability to actively adsorb leukocytes to their surfaces. This process is now known to be mediated by adhesion molecules. Over a decade ago, technology was developed to isolate and cul-

ture vascular endothelial cells and airway epithelial cells, leading to tremendous advances in our understanding of adhesion molecules and their importance in a variety of normal and pathological responses. The past decade has also witnessed a flood of information on cytokines and chemokines and their effects on adhesion molecules in inflammation. Indeed, most of these molecules were not even known to exist until a few years ago. For adhesion molecules, this new knowledge includes characterization of their molecular structures and counter-ligands, classifications into superfamilies, investigations into signal transduction events, and analyses of the regulation of their expression and function both in vitro and in vivo.

The overall aim of this book is to update the reader on the cells, proteins, and mechanisms involved in allergic inflammation, with a major emphasis on mechanisms of local cell recruitment. With this in mind, the first five chapters present overviews of adhesion molecule biology, familiarizing the reader with the latest lists of adhesion molecules, their nomenclature, and general biological functions. Included are chapters on specific types of adhesion molecules (e.g., integrins), as well as chapters on the adhesive capabilities of endothelial cells, respiratory epithelial cells and homing to mucosal surfaces. The remaining chapters have a more narrow focus. Seven chapters cover adhesion-related biology of cell types felt to be particularly important in allergic inflammatory reactions, including mast cells, basophils and eosinophils. These chapters primarily discuss findings from in vitro studies and summarize cell adhesion phenotype and functional endothelial, epithelial, and extracellular matrix protein ligands for each cell type. Also covered are the effects of adhesion on cell function, and regulation of adhesion molecule expression and function. In the final eight chapters, adhesion molecule expression and function in vivo in various allergic and other immune responses are covered, and, where available, data on adhesion molecule antagonism are presented.

Although other reviews of adhesion molecules have appeared, this is the first book to be devoted exclusively to allergic inflammation. Its publication seems especially timely in that efforts are now underway, in both animals and humans, to antagonize the function and/or expression of these adhesion molecules as therapeutic targets, in an attempt to generate novel anti-inflammatory treatments of allergic disease. Hopefully, future editions of this book will be able to incorporate new information in this area, as well as data in other areas where information is lacking, such as the trafficking of monocytes and macrophages during allergic inflammation.

As editor of this book I am indebted to the contributors, all of whom are experts in their respective fields, and without whose contributions this text would not have been possible. I would also like to thank my family for their never-ending love and support and my mentor and long-time collaborator, Dr. Robert Schleimer, who helped to foster my intense interest in the field, and with whom I

have been fortunate to share the excitement and evolution of our work. My thanks to the past and present members of the "Schlochneck" laboratory group for their hard work, ambition, inquisitiveness and enthusiasm, which makes research fun, the staff at Marcel Dekker, Inc., for continual assistance throughout this project, and Bonnie Hebden for excellent administrative and secretarial support.

Bruce S. Bochner

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15 Endothelial Cells and Cell Adhesion

BRUCE S. BOCHNER ROBERT P. SCHLEIMER

During inflammatory reactions, tissue-resident cells generate signals that activate cellular and fluid elements in the intravascular compartment, resulting in the initiation of a cascade of events leading to leukocyte recruitment. During this process, the vascular endothelium was initially believed to play only a passive barrier role, the integrity of this barrier becoming altered to allow the influx or leakage of cells and plasma into the site. The concept that the vascular endothelium could itself be actively involved in local inflammation site was initially suggested in the late 1800s by studies that detected histologic changes in endothelial cells at inflammatory sites.[104] Subsequently, animal models of inflammation utilizing techniques such as intravital microscopy revealed that local tissue injury caused circulating cells to rapidly marginate onto the adjacent luminal surface of the vasculature.[11] However, one of the most significant advances in the field of endothelial biology occurred when techniques were developed to isolate and culture vascular endothelial cells from sources such as human umbilical veins[171, 224] and dermal microvascular sites.[illegible] Analysis of cultured endothelial cells led to dramatic improvements in our understanding of the role of the endothelial cell in a variety of normal and pathologic responses, including angiogenesis, atherogenesis, wound healing, tumor metastasis, coagulation, and leukocyte recruitment during inflammation.

Occurring in parallel with these latter studies were discoveries related to leukocyte surface structures involved in cell migration and attachment. From these investigations emerged novel areas of research focusing on cell adhesion molecules and their role in inflammation. Adhesion molecules are now known to be critical for virtually every step in cell recruitment, including margination, diapedesis, and chemotaxis. Within the past decade, tremendous growth has occurred in our knowledge of these ever-increasing families of molecules. More than 20 adhesion molecules have now been identified and cloned. A variety of adhesion molecule knock-out mice have been created, and adhesion molecule antagonists have been developed that are now being tested in vivo. These and other studies have contributed greatly to our understanding of the biologic importance and relative contributions of these molecules in a variety of immunologic responses.

The goal of this chapter is to summarize the function of endothelial cells and leukocytes during human inflammatory reactions, especially those events mediated by cell-cell contact through adhesion molecules. Many aspects of endothelial cell biology cannot be covered but fortunately have been summarized elsewhere. For example, the embryologic, ultrastructural, and morphologic characteristics of endothelial cells have been the subject of several excellent texts.[348, 434–436, 473] Similarly, the important functions of endothelium in regulating blood flow and coagulation processes have been discussed elsewhere.[313, 434, 498, 534]

This chapter reviews several aspects of human endothelial cell biology and function, beginning with the role of the endothelial cell as a source of inflammatory mediators as well as potential interactions between endothelial cells and adjacent mast cells. This review is followed by a more extensive discussion of leukocyte-endothelial interactions mediated by cell adhesion molecules. Molecular and biologic aspects of adhesion molecule function and expression are considered, and current knowledge regarding in vivo expression and function of these structures is highlighted. Because of their expansive nature, discussions of most of these topics are restricted to those cells and molecules most relevant to allergic inflammation.

REGULATION OF ENDOTHELIAL PERMEABILITY AND PROLIFERATION BY MAST CELLS

The maintenance of vascular integrity is one of the most essential functions of endothelium. Several mast cell–derived

mediators, including histamine and arachidonic acid metabolites, can cause increases in vascular permeability,[243] apparently through endothelial cell contraction. The precise mechanisms by which vascular permeability is regulated remain unclear.[166, 348] For histamine, increased permeability is believed to be mediated through an interaction with endothelial H_1 and H_2 cell surface receptors.[472] In addition to its effects on endothelial permeability, histamine rapidly induces the surface expression of the adhesion molecule P-selectin (CD62P), which exists preformed within the Weibel-Palade bodies that also contain von Willebrand factor.[298] Endothelial cells also express neurokinin-1 receptors,[181] which may be responsible for substance P–induced changes in vascular permeability.

The interaction between mast cells and endothelium, however, involves more than the regulation of vascular permeability by mast cell mediators. Several lines of evidence indicate that mast cell heparin can stimulate endothelial cell proliferation and that endothelial cell products can, in turn, stimulate mast cell proliferation. It has clearly been demonstrated that mast cell numbers increase dramatically at sites of angiogenesis such as in solid tumors,[242] and more perivascular mast cells are found in skin biopsy samples of subjects with urticaria.[363] Mast cell heparin stimulates endothelial cell proliferation by physical association with endothelial cell growth factor,[523] increasing the potency of the latter approximately 30-fold.[453] The importance of mast cell heparin in angiogenesis is further suggested by its ability to induce endothelial cell migration.[24] In addition to mast cell–derived substances, other factors, including vascular endothelial growth factor[254] and IL-4,[426] may regulate endothelial migration and proliferation. Mast cells may be a source of cytokines, such as TNF, IL-4, and IL-13,[71, 78, 252, 552] which are capable of activating endothelial adhesion molecule expression.

VASCULAR ENDOTHELIAL CELLS AS ANTIGEN-PRESENTING CELLS

Classically, monocytes and macrophages have been considered the primary cells responsible for antigen presentation, but there is evidence that under certain conditions vascular endothelial cells can perform accessory cell functions. Human umbilical vein and dermal endothelial cells can process and present a variety of soluble antigens to T cells.[106, 403, 545] They can support allogeneic, mitogen-stimulated, and antigen-stimulated T-cell responses.[401] As is the case with macrophages, expression of class II antigens (e.g., HLA-DR and HLA-DS) by endothelial cells can be induced by lymphokines such as gamma interferon.[106] Endothelial cells possess other characteristics of antigen-presenting cells such as the ability to express cell surface receptors for C3b and IgG.[437]

VASCULAR ENDOTHELIAL CELLS AS A SOURCE OF CHEMICAL MEDIATORS AND SOLUBLE PROTEINS

Vascular endothelial cells produce a variety of factors essential to supporting homeostasis throughout the human body.[313, 534] Endothelial cell products important for the maintenance of hemostasis and blood flow include Factor VIII–related antigen,[213] plasminogen activator,[272] thromboplastin,[225] endothelin-1,[534, 535] C1 esterase inhibitor,[452] and angiotensin-converting enzyme.[123] Endothelial cells respond within minutes to stimulation with ionophore, thrombin, bradykinin, histamine, and sulfidopeptide leukotrienes by producing platelet-activating factor (PAF) and prostacyclin, mediators that promote leukocyte activation, vasodilation, and increased vascular permeability.[26, 87, 324, 325, 407, 594] In contrast to the rapid effects of these stimuli on vascular endothelial cells, responses to other agents occur more slowly. For example, IL-1 treatment of vascular endothelial cells leads to the production of PAF (including or exclusively the acyl form) and prostacyclin; but in contrast to the immediate production of these mediators in response to thrombin or histamine, this effect occurs only after a delay of several hours.[79, 426, 530] Activated endothelial cells also produce nitric oxide[388]; matrix proteins, including fibronectin[213]; several cytokines, including IL-1,[497] IL-6,[125, 374] and platelet-derived growth factor[8]; several C-X-C chemokines such as IL-8,[172] groα,[130] and IP-10[308]; several C-C chemokines such as RANTES[314, 494] and MCP-1[420, 468]; and a variety of colony-stimulating factors, including G-CSF, M-CSF, and GM-CSF,[27, 75, 269, 429, 458] which are capable of activating eosinophils[268, 269, 296, 527] as well as endothelial cells themselves.[80, 81] These and potentially other mediators derived from endothelium further illustrate the active role of the vascular endothelium in inflammatory responses.

MOLECULAR ASPECTS OF ADHESION MOLECULES

Many cell surface structures on endothelial cells and leukocytes are capable of mediating adhesion. These molecules are subdivided into families (integrins, immunoglobulin-like structures, selectins, and carbohydrate counterligands for selectins) based on shared structural characteristics and functions. This section describes these structural characteristics on a molecular and biochemical level, as well as various phenotypic and functional aspects of these molecules in vitro. Several additional reviews on these topics have also appeared.[44, 89, 217, 218, 275, 401, 484]

INTEGRINS

The integrin family consists of more than 15 transmembrane, noncovalently associated heterodimers with distinct α and β chains that are responsible for adhesion to other cell surface ligands, complement protein fragments, and extracellular matrix proteins; nearly all have been cloned.[6, 203, 432] These molecules, in addition to mediating adhesion, have important signaling functions.[218, 421, 446, 455] So far, at least 15 α subunits and 8 β subunits have been identified, with 20 heterodimeric pairings documented to date. Although it was initially believed that α and β subunit pairings were restricted according to the β subunits, it is now clear that different α subunits can associate with more than one β subunit.[21]

The structure of a typical integrin is shown schematically in Figure 15–1. The α subunits range in size from 120 to

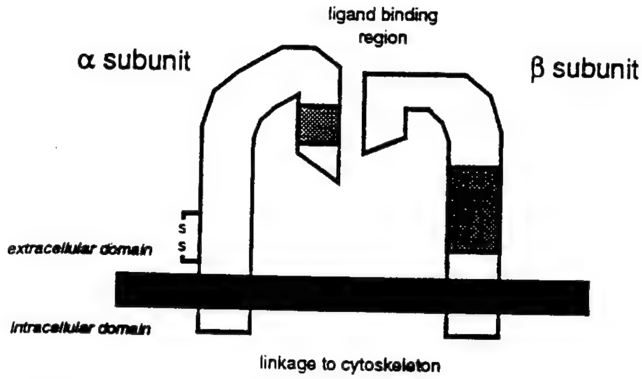

FIGURE 15–1. Basic structure of an integrin heterodimer. The divalent cation binding region on the α subunit and the cysteine-rich repeat region on the β subunit are shown as shaded areas. Some α subunits contain an inserted (I) domain within the ligand binding region located membrane proximal to the cation binding site.

210 kD, and the β subunits range from 90 to 110 kD. Overall, there is a higher degree of homology among β subunits than among α subunits.[247] Characteristic features of the intracytoplasmic domains of these subunits include sites for phosphorylation[45, 96, 208] as well as for attachment to cytoskeletal elements such as talin, vinculin, α-actinin, filamin, and actin.[462, 474] During cell-substratum adhesion, integrins, along with these cytoskeletal proteins, tend to accumulate in patches called focal contacts.[117, 474] A conserved sequence in the cytoplasmic carboxyl terminus of several β subunits, separate from the phosphorylation sites, appears to influence the avidity of binding.[208, 209] Within the extracellular portions of α subunits are three or four domains, each approximately 60 amino acids in length, that resemble calcium-binding sites found in other proteins. By binding divalent cations (typically calcium and magnesium), these domains are believed to contribute to the binding affinity of integrin heterodimers.[35, 210] Another interesting characteristic of some integrins (all of the α chains for the β2 integrins as well as the α1 and α2 chains of β1 integrins) is the presence of an inserted, or I, domain, belonging to a large family of A domain proteins.[107] This site appears to be an important recognition site for integrin-binding activity.[127, 271] A characteristic feature of the extracellular portions of β subunits, unlike α subunits, is the presence of 56 conserved cysteine residues, localized primarily to four tandem domains; their presence is believed to contribute to the rigidity of these molecules.[250]

Umbilical vein endothelial cells express several β1 integrins (α2β1, α3β1, α5β1, α6β1) as well as αvβ3 (Table 15–1)[54]; microvascular endothelial cells may express additional integrins, albeit at relatively low levels.[580] Since integrins can be expressed on both the basal and luminal surfaces of endothelial cells,[7, 109] these receptors are believed to mediate adhesion to substratum as well as to intraluminal ligands. On eosinophils, among the β1 integrins, α4β1 and α6β1 are expressed,[145, 170] whereas basophils and mast cells express α4β1 and α5β1 (Table 15–2).[51, 188, 532] Other integrin subfamilies are restricted to certain cell types. An example of this is the β2 integrins, whose expression is essentially limited to leukocytes.[250] Among different cell types, however, levels of surface expression vary, and the levels of cell surface expression can be altered during hematopoiesis[245, 547] or as a consequence of cellular activation. For example, initial studies of the induction of expression of VLA (very late activation) proteins, a subfamily of integrins now known to represent the β1 integrins, demonstrated that prolonged activation of lymphocytes was required in vitro with mitogens before these structures became expressed.[203]

Other integrins, such as Mac-1 (αMβ2, CD11b/CD18), exist both on the cell surface and in an intracytoplasmic pool of granules, which can rapidly translocate to the cell surface following activation with agents such as chemotactic factors or eosinophil proteins.[349, 459] Whereas chemotactic factors such as fMLP, PAF, and C5a induce up-regulation of Mac-1 on both eosinophils and neutrophils, IL-5 can selectively affect Mac-1 expression on eosinophils.[303, 367, 549] However, in addition to the level of adhesion molecule expression, it is now apparent that conformational changes can occur in integrins,[105] resulting in rapid, reversible changes in binding avidity.[128, 307, 481] These changes occur as a result of ligand binding,[22, 210, 257, 258] occupancy of divalent cation binding sites,[13, 463] or allosteric changes caused by adjacent cell surface structures such as integrin-modulating factor-1[206] or in association with phosphorylation (e.g., via focal adhesion protein-tyrosine kinase) of clustered intracytoplasmic domains of the integrin subunits.[93, 239, 377, 412, 446, 588]

Expression of integrins appears also under transcriptional regulation, and analyses of the promoter sequences of several integrin genes have identified myeloid transcription factors (e.g., PU-1) that influence gene expression.[157, 386, 387, 424, 425] Intracytoplasmic assembly and subsequent expression of integrin heterodimers appear to require an intact β subunit, since genetic mutations in the β2 subunit (especially near the *N*-terminal portion) have been identified in patients with a disorder called leukocyte adhesion deficiency disease type 1, in which leukocyte surface expression of β2 integrins is markedly impaired or totally absent.[16, 21] Indeed, the defect can be corrected by gene transfer.[576]

The functional ligands for integrins expressed on endothelial cells, leukocytes, platelets, and mast cells are listed in Tables 15–1 and 15–2. As a rule, integrins belonging to the β1 family are ligands for extracellular matrix proteins, such as collagen, laminin, and fibronectin, and mediate firm attachment and spreading of cells under static conditions.[464] The integrin α2β1 also functions as a ligand for echovirus.[40, 41] The VLA-4 heterodimer (CD49d/CD29, α4β1) is of particular interest in allergic inflammation.[62] It binds both to the CS-1 (connecting segment-1) portion of the IIICS (type III connecting segment) region of fibronectin (containing the consensus amino acid sequence LDV)[543, 562, 563] and to the regions within the first and fourth domains of VCAM-1 (vascular cell adhesion molecule-1), a molecule expressed on activated endothelial cells (Fig. 15–2).[150, 385, 391, 543, 544]

As with other integrins, expression of VLA-4 is under transcriptional regulation.[422] Several studies suggest that the avidity of VLA-4 for its ligands differs among cell types and can be dramatically altered by cell activation.[94, 95, 211, 318, 410, 441] Other characteristics unique to VLA-4 are its lack of expression on neutrophils, despite broad expression on all other leukocytes,[57, 204] and its ability to mediate adhesion under conditions of shear stress, often referred to as rolling adhesion.[12, 306, 486] This function is usually considered one in which selectins play a more important role. Another β sub-

TABLE 15–1
BIOCHEMICAL AND FUNCTIONAL CHARACTERISTICS OF ENDOTHELIAL ADHESION MOLECULES

Adhesion Molecule	CD	Size (kD)	Expression and/or Inducing Stimuli	Ligands
Integrins				
α2β1 (VLA-2)	CD49b/CD29	160/130	Constitutive	Collagen, laminin, echovirus 1
α3β1 (VLA-3)	CD49c/CD29	150/130	Constitutive	Laminin, fibronectin, collagen
α5β1 (VLA-5)	CD49e/CD29	160/130	Constitutive	Fibronectin
α6β1 (VLA-6)	CD49f/CD29	150/130	Constitutive	Laminin
αVβ3	CD51/CD61	165/105	Constitutive	Vitronectin, fibrinogen, fibronectin, others
Immunoglobulin Gene Superfamily				
ICAM-1	CD54	100	Constitutive, IL-1, TNF, LPS, IFN-γ	CD11a/CD18, CD11b/CD18, rhinovirus
ICAM-2	CD102	60	Constitutive	CD11a/CD18
PECAM-1	CD31	125	Constitutive	CD31
VCAM-1	CD106	100	IL-1, TNF, LPS, IL-4, IL-13	CD49d/CD29 (α4β1) and α4β7
MAdCAM-1*	None	60	Constitutive	α4β7, CD62L (L-selectin)
Selectins				
E-selectin	CD62E	110	IL-1, TNF, LPS	sLe[x], sLe[a], CLA, s-di-Le[x], ESL-1, myeloglycans
P-selectin	CD62P	140	Histamine, thrombin, C5a, peroxides, phorbol esters, ionophores	Le[x], sLe[x], sLe[a], PSGL-1, sulfated glycolipids
Others				
Pgp-1 (Hermes)	CD44	90	Constitutive	Hyaluronate, collagen
VAP-1*	None	90	Constitutive	Unknown lymphocyte structure
L-VAP-2	CD73	70	Constitutive	Unknown lymphocyte structure

*Expressed primarily or exclusively on lymphoid high endothelial venules.

TABLE 15–2
BIOCHEMICAL AND FUNCTIONAL CHARACTERISTICS OF ADHESION MOLECULES ON HUMAN LEUKOCYTES, MAST CELLS, AND PLATELETS

Type	Name (CD)	Size (kD)	Ligands	Cellular Distribution
Integrins				
β1 (VLA) family	α1β1 (CD49a/CD29)	210/130	Laminin, collagen	L
	α2β1 (CD49b/CD29)	160/130	Collagen, laminin	L, M, P
	α3β1 (CD49c/CD29)	150/130	Collagen, laminin, others	L
	α4β1 (CD49d/CD29)	150/130	VCAM-1, fibronectin	L, M, E, B, MC
	α5β1 (CD49e/CD29)	160/130	Fibronectin	L, M, N, B, MC, P
	α6β1 (CD49f/CD29)	150/130	Laminin	L, M, N, E, P
β2 family	LFA-1 (CD11a/CD18)	180/95	ICAM-1, ICAM-2, ICAM-3	L, M, N, E, B
	Mac-1 (CD11b/CD18)	170/95	C3bi, ICAM-1, fibrinogen	L, M, N, E, B
	p150, 95 (CD11c/CD18)	150/95	C3bi, others	L, M, N, E, B, MC*
	αdβ2 (αd/CD18)	125	ICAM-3	L, M, N, E, B
β3 family	αIIbβ3 (CD41/CD61)	120/23/105	Fibrinogen, fibronectin, others	P
	αvβ3 (CD51/Cd61)	163/105	Vitronectin, others	M, MC, P
Other β integrins	α4β7	150/120	MAdCAM-1, VCAM-1, fibronectin	L, M, E, B
	αEβ7	150/25	E-cadherin	L
Immunoglobulin Gene Superfamily				
	ICAM-1 (CD54)	100	LFA-1, Mac-1	L, M, N, B, MC
	ICAM-2 (CD102)	60	LFA-1	L, M, B, MC, P
	ICAM-3 (CD50)	124	LFA-1	L, M, N, E, B, MC
	PECAM-1 (CD31)	125	CD31	L, M, N, E, B, P
	LFA-3 (CD58)	70	CD2	L, M, N, E, B, MC, P
Selectins				
	L-selectin (CD62L)	80	GlyCAM-1, CD34, MAdCAM-1	L, M, N, E, B
	P-selectin (CD62P)	150	PSGL-1, SLe[x]	P
Carbohydrates and Others				
	Lewis[x] (Le[x], CD15)	Unknown	P-selectin	M, N, E
	sLe[x] (CD15s)	Unknown	E-selectin, P-selectin	M, N, E, B
	s-dimeric Le[x]	Unknown	E-selectin	M, N, E, B
	Pgp-1/Hermes (CD44)	90	Hyaluronate, collagen	L, M, N, E, B, MC

*Weakly expressed on uterine mast cells but not mast cells from other tissues.
Abbreviations: L = lymphocytes; M = monocytes; N = neutrophils; E = eosinophils; B = basophils; MC = mast cells; P = platelets.

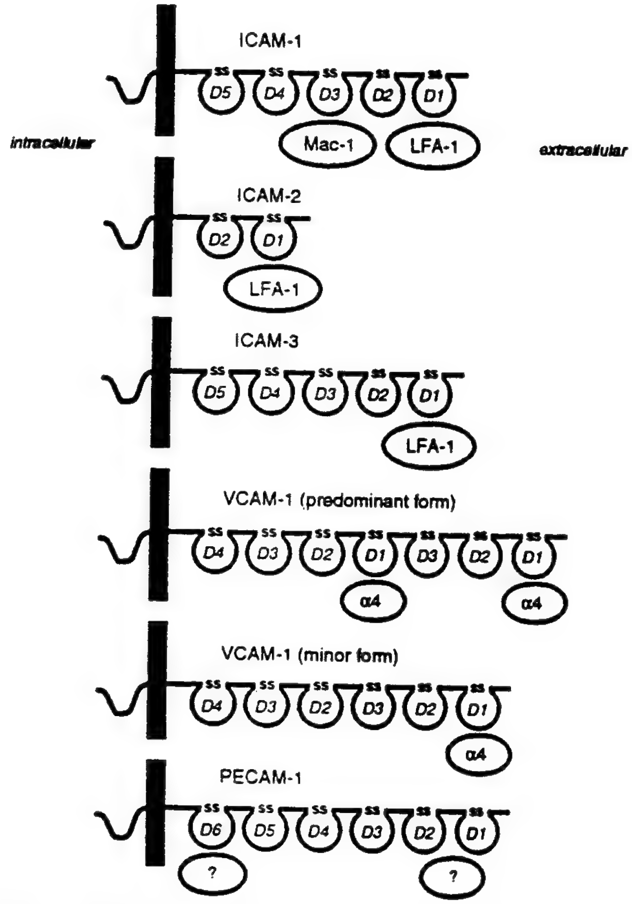

FIGURE 15–2. Basic structures of several immunoglobulin gene superfamily molecules expressed on endothelial cells. Cellular counterligands (ovals) are shown adjacent to the domains (D) containing sequences recognized during binding. The exact counterligands and binding sites on PECAM-1 are unknown. However, mAb that map to domains 1 and 2 block homotypic (PECAM-1 to PECAM-1) adhesion and leukocyte transendothelial migration, and mAb that map to domain 6 block heterotypic adhesion.

unit, β7, can also pair with α4 (α4β7) and, like VLA-4, is capable of binding to fibronectin and VCAM-1.[406, 431] Unlike VLA-4, however, α4β7 binds to another adhesion molecule, MAdCAM-1 (mucosal addressin cell adhesion molecule-1), that appears to be involved in lymphocyte homing to the gut mucosa.[17, 42, 73, 153, 456] Furthermore, β7 can pair with an additional subunit, αE, which is expressed on lymphocytes (but not granulocytes), where it functions as a ligand for E-cadherin, a molecule found along the basolateral portion of intestinal epithelium.[91, 92]

Ligands for β2 integrins include ICAM-1, ICAM-2, and ICAM-3, as well as fibrinogen, the complement fragment C3bi, and other unidentified structures (see Table 15–2 and Fig. 15–2).[21, 49, 119, 127, 223, 315, 487, 490] For all leukocytes, the processes of firm adhesion, locomotion, and transendothelial migration, as seen in response to stimulation with chemotactic factors, are either partially or completely dependent on β2 integrins. Defects in β2 integrin expression lead to impaired leukocyte recruitment responses, especially in neutrophils.[16, 21]

IMMUNOGLOBULIN GENE SUPERFAMILY

The immunoglobulin gene superfamily of adhesion molecules consists of more than a dozen molecules that have a series of globular domains, formed by disulfide bonds, resembling those found in immunoglobulins.[141] Like integrins, these molecules are responsible for adhesion to other cell surface ligands and have important signaling functions. Members of this family expressed on endothelial cells include ICAM-1, ICAM-2, PECAM-1 (platelet-endothelial cell adhesion molecule-1), VCAM-1, and MAdCAM-1, whereas leukocytes can express ICAM-1, ICAM-2, ICAM-3, PECAM-1, and LFA-3 (CD58) (see Tables 15–1 and 15–2).

The structures of ICAM-1, ICAM-2, ICAM-3, VCAM-1, and PECAM-1 are shown schematically in Figure 15–2; each is discussed in turn. ICAM-1 (CD54) was originally discovered as a ≈90 kD molecule responsible for heterotypic cell adhesion, with a 453 amino acid extracellular domain and putative 24 and 28 amino acid transmembrane and intracytoplasmic domains, respectively.[430, 491] Ligands for the first *N*-terminal domain of ICAM-1 include the β2 integrin LFA-1, fibrinogen, and most serotypes of rhinovirus,[182, 273, 315, 489] whereas the third domain is recognized by the β2 integrin Mac-1.[129] ICAM-1 is constitutively expressed along the luminal, intercellular, and subluminal surfaces of endothelial cells.[383] Various stimuli, including IL-1, TNF, LPS, and IFN-γ, are capable of inducing or enhancing its expression, primarily at the level of transcription (Table 15–3).[142, 546] Unique to IFN-γ is its ability to selectively induce ICAM-1 expression without affecting expression of other adhesion molecules (see Table 15–3).[142, 402] ICAM-1 expression can be induced on several leukocyte types (e.g., eosinophils and basophils)[195, 532] as well as other cells, including respiratory and ocular epithelial cells.[14, 102, 528]

As the name implies, ICAM-2 (CD102) is similar to ICAM-1. It was originally detected as an LFA-1–dependent, ICAM-1–independent 60-kD endothelial ligand with a 202 amino acid extracellular domain and putative transmembrane and intracytoplasmic domains of 26 amino acids each.[120, 490] ICAM-2 has two immunoglobulin-like extracellular domains

TABLE 15–3
EFFECTS OF CYTOKINES ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELL EXPRESSION OF VCAM-1, ICAM-1, AND E-SELECTIN

	Level of Cell Surface Expression*		
Endothelial Treatment†	***VCAM-1***	***ICAM-1***	***E-Selectin***
None	−	+	−
IFN-γ, 24 hr	−	++	−
IL-1, TNF, or LPS, 4–6 hours	++	+++	++++
IL-1, TNF, or LPS, 24 hours	+++	++++	+
IL-4 or IL-13, 4–6 hours	+	+	−
IL-4 or IL-13, 24 hours	++	+	−
IL-4 + TNF, 24 hours	++++	+++	+/−
IL-4 + TNF (low dose), 24 hours	+++	+	−

*Levels of expression, as determined by flow cytometry, are given on a scale of − (absent) to ++++ (maximal). Note that maximal levels of expression differ among these surface structures (ICAM-1>E-selectin>VCAM-1).
†Optimal concentrations for endothelial activation: IFN-γ (10 ng/ml), IL-1 (1 ng/ml), TNF (1 ng/ml or 0.03 ng/ml (low dose)), LPS (bacterial endotoxin, 1 μg/ml), IL-4 (500 units/ml), and IL-13 (10 units/ml).

with 34% homology to the first two domains of ICAM-1.[490] As with ICAM-1, LFA-1–mediated adhesion to ICAM-2 can serve as a costimulatory signal for lymphocyte proliferation.[114] The ligand-binding site for LFA-1 is located in the first *N*-terminal domain in ICAM-1; peptides from this region have been shown to inhibit endothelial cell adhesion.[291] ICAM-2 is constitutively expressed on mononuclear cells, basophils, mast cells, and platelets; endothelial cells, which also express ICAM-2, appear to be the only other cell type that expresses this molecule.[120, 376] ICAM-2 expression is unaffected by cytokines in vitro,[375] although increased levels have been detected in endothelial cells from malignant lymph nodes, suggesting possible regulation of cellular expression in vivo.[414]

ICAM-3 (CD50) also functions as an LFA-1 ligand.[119, 156, 229, 538] It ranges in molecular weight from 116 to 140 kD, depending on the cell type studied, and possesses 48% to 52% homology to ICAM-1 and 31% to 37% homology to ICAM-2.[118, 156, 538] ICAM-3, like ICAM-1, has five immunoglobulin-like extracellular domains (518 amino acids), along with a transmembrane domain of 24 amino acids and an intracytoplasmic domain of 37 amino acids.[118, 156, 538] ICAM-3 is constitutively expressed on all leukocytes and on mast cells; expression on other cell types, including endothelial cells, has not been detected.[119, 156, 532, 538] ICAM-3 appears to act as a signaling molecule, since cross-linking results in calcium mobilization, tyrosine phosphorylation, and adhesion.[101, 230] Following neutrophil activation, the molecule can be proteolytically released from the cell surface.[122]

VCAM-1 (CD106), which is not constitutively expressed on endothelium, was initially identified as a cytokine-inducible structure on endothelial cells that contains six immunoglobulin domains.[384] However, it was later determined that it exists primarily in a seven domain form where there is extensive homology between the three most *N*-terminal domains (labeled domains 1, 2, and 3) and the fourth through sixth domains, suggesting that the molecule developed through gene duplication.[112, 113, 404] In fact, the smaller, six domain form, lacking domain 4, is expressed at very low levels, presumably representing an alternatively spliced form of the molecule.[112, 113, 207, 404] Both the six and seven domain forms have type 1 transmembrane polypeptide anchors consisting of a 22 amino acid transmembrane domain and an intracytoplasmic region of 19 residues.[112, 113, 207, 384, 404] An even smaller glycophosphatidylinositol (GPI)-anchored isoform of VCAM-1 has been detected in murine endothelium.[246, 517] Within the extracellular portions of VCAM-1, domains 1 and 4 are most homologous to each other; these are the domains recognized by VLA-4,[385, 391, 543, 544] although the third domain is needed to stabilize these binding sites.[564]

Although it is usually considered an endothelial cell surface marker, VCAM-1 expression has been detected on other cell types, including macrophages, dendritic cells, astrocytes, and stromal cells in bone marrow.[417, 423] Expression of VCAM-1 on umbilical vein endothelial cells is concentrated on the luminal surface[383] and can be induced de novo within several hours after exposure to interleukin-1 (IL-1), tumor necrosis factor (TNF), or bacterial endotoxin (LPS); expression reaches maximal levels by 24 to 48 hours (see Table 15–3).[117, 384, 416, 573] These treatment conditions lead to increased expression of other endothelial adhesion molecules, including ICAM-1 and E-selectin (see Table 15–3). In contrast, treatment of endothelial cells with IL-4[451, 522] or IL-13[55, 476] leads to selective induction of VCAM-1 expression, and the combination of IL-4 with TNF is synergistic.[316, 521] This effect is due to transcriptional activation and stabilization of VCAM-1 mRNA.[219] Molecular analyses of the VCAM-1 promoter and cell signaling events suggest that NF-κB and protein kinase C are involved in the induction of VCAM-1 expression caused by some cytokines.[121, 368, 467] These patterns of activation may not necessarily be true for other endothelial cell types. For example, human dermal microvascular endothelial cells express VCAM-1 after stimulation with TNF but not after stimulation with either IL-1 or IL-4.[503]

PECAM-1 (platelet-endothelial cell adhesion molecule-1) is a 130-kD molecule with six immunoglobulin-like domains.[124, 351, 370, 499] Unlike other members of this adhesion molecule family, the transmembrane and intracytoplasmic domains are encoded by multiple exons, and several isoforms, due to alternative splicing within these regions, have been identified.[124] As its name implies, PECAM-1 is constitutively expressed on endothelial cells and platelets, although most leukocyte types also express this molecule.[499, 532] The molecule is rapidly shed following activation with chemotactic factors.[499] Both homotypic[9] and heterotypic[352] adhesion via PECAM-1 have been reported, an example of the latter being the interaction of CD31 with sulfated glycosylaminoglycans such as heparan sulfate. Blocking monoclonal antibodies that recognize the second domain are capable of interrupting heterotypic interactions,[124, 351] whereas antibodies to domains 5 and 6, along with antibodies to domains 2 and 3, are required to interrupt homotypic binding, suggesting antiparallel interactions.[155] Cross-linking of PECAM-1 augments avidity of both β1 and β2 integrins.[43, 155, 393, 509]

SELECTINS AND THEIR CARBOHYDRATE LIGANDS

Another family of adhesion molecules is the selectin gene superfamily.[45, 275, 299] The only three known members, E-selectin, L-selectin, and P-selectin, are now referred to as CD62 followed by their respective first letters (CD62E, CD62L, and CD62P). E-selectin (formerly endothelial-leukocyte adhesion molecule-1 or ELAM-1, 115 kD)[48] is expressed exclusively on activated endothelium. P-selectin (formerly GMP-140 or PADGEM, 150 kD),[227] the largest selectin, originally received its name because of its stimulus-induced expression on platelets. It can also be expressed on the surface of endothelial cells. L-selectin is the smallest selectin (formerly TQ1, LECCAM-1, LECAM-1, Leu-8, or LAM-1, 75 kD on lymphocytes, 100 kD on granulocytes, and 110 kD on monocytes)[89, 511] and gets its name because of its restricted expression on leukocytes. It is now believed that the major function of selectins in vivo is to mediate leukocyte-endothelial interactions under conditions of shear stress; L-selectin also functions during lymphocyte trafficking to lymph nodes.[44, 45, 82, 275]

The structures of the selectins are shown schematically in Figure 15–3. Each consists of an *N*-terminal domain of 117 to 120 amino acids possessing calcium-dependent (C type) lectin activity.[137] Proximal to this region is a 32 to 38 amino acid segment with homology to a domain initially discovered

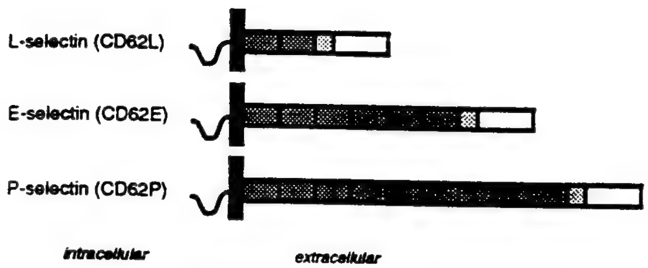

FIGURE 15–3. Basic structures of selectins. The *N*-terminal lectin domains are shown in white, the epidermal growth factor-like domains are shown in light gray, and the complement regulatory-like repeat domains, which vary in number among the different selectins, are shown in dark gray.

in epidermal growth factor, which is referred to as the EGF domain. Proximal to this are two to nine domains of about 60 amino acids in length whose sequences resemble those found in both soluble and membrane-bound complement regulatory (CR) proteins such as CD35 (type 1 complement receptor), decay-accelerating factor (CD55), C1r, C1s, and C4-binding protein.[48, 233] Of note, the genes for selectins are all located on chromosome 1, near the genes for these and other CR proteins.[560] These sequences are then followed by short transmembrane and intracytoplasmic domains of 21 to 35 amino acids each. Although the extracellular domains share significant homology (40–60 overall and 60%–70% within the lectin and EGF domains), little if any homology exists among the transmembrane and intracytoplasmic domains.[275] For P-selectin, two variant forms have been identified: one lacking the transmembrane and intracytoplasmic portions (probably representing a soluble form) and a second alternatively spliced form with eight CR subunits instead of nine.[138, 226] For cell-cell adhesion, the functional portions of all selectins appear to be restricted to the lectin domains, although the presence of the EGF domain may be necessary to confer the appropriate tertiary conformation for binding.[151, 152, 179, 238, 275, 390] For L-selectin, at least, the intracytoplasmic portion of the molecule is also required for adhesive function.[237] Adhesion via selectins or their ligands can alter integrin function as well.[262, 294, 302, 542]

E-selectin is not present on the surface of resting endothelium. Expression of E-selectin is inducible within several hours in cultured endothelial cells, in tissue explants, and/or at injection sites after exposure to various stimuli, including IL-1, TNF, LPS,[46, 234, 329, 470] substance P,[319] IL-3,[74] and uncharacterized substances from platelets.[192] Expression can be potentiated by IFN-γ[284] and inhibited by transforming growth factor β.[164, 165] Once expressed, E-selectin can function as a ligand for leukocytes, including neutrophils,[47, 158, 450] monocytes,[47] eosinophils,[57, 84, 266, 572] basophils,[57, 60] NK cells,[400] and subsets of T lymphocytes.[177, 394, 466] Other cell types, including tumor cells, also recognize E-selectin.[311, 505] Molecular studies of the E-selectin promoter have revealed that transcription is under the control of several transcription factors, including NF-κB.[126, 139] Expression of E-selectin in vitro is relatively transient, with levels approaching those at baseline by 24 hours,[46, 55] although its expression can be prolonged in vitro by incorporating several incompletely defined human plasma-derived factors in the culture.[460] Most of the E-selectin that is expressed is reinternalized and degraded in vitro,[265] but a small amount can be recovered from the culture supernatant, perhaps due to shedding.[283, 371, 398] In vivo, however, E-selectin expression at sites of inflammation is more prolonged,[111, 184] perhaps due to differences in posttranscriptional stability among forms of E-selectin transcripts.[99]

Like E-selectin, P-selectin is not present on the luminal surface of resting endothelium. However, unlike E-selectin, P-selectin exists preformed within granules (the so-called Weibel-Palade bodies)[68, 199, 323, 496] and can be rapidly expressed (within minutes) after stimulation with agents such as histamine, thrombin, phorbol esters, peroxides, and C5a.[159, 167, 389] Expression of P-selectin in vitro is downregulated as a consequence of endocytosis.[199, 297] P-selectin has been shown to be a ligand for many cell types, including neutrophils, eosinophils, monocytes, and some T lymphocytes.[115, 167, 341, 571] Leukocyte interaction with P-selectin has been shown to alter cellular functions, including superoxide production and integrin-mediated phagocytosis.[110, 531, 578]

The third and smallest member of the selectin family, L-selectin, is found exclusively on leukocytes.[288, 512] It was originally discovered in mice as the peripheral lymph node homing receptor, a molecule responsible for lymphocyte attachment to high endothelial venules.[37, 70, 86, 162, 168, 249, 469, 488] It also functions as an adhesion molecule for vascular endothelium under conditions of shear stress.[35, 253, 306, 482, 483, 541, 542] L-selectin is shed through an undefined proteolytic pathway during leukocyte activation by chemotactic factors, cytokines, and other stimuli.[39, 183, 231, 236, 248, 249, 512]

A great deal of effort has focused on identifying the carbohydrate ligands for selectins.[299, 537] Studies have examined the carbohydrates themselves, the core structures upon which the carbohydrates are expressed, as well as the enzymatic pathways responsible for their synthesis. At first glance, the interactions between selectins and their ligands appear quite similar and difficult to distinguish. In fact, many binding characteristics are shared, including calcium dependence, function at low temperatures and under conditions of shear stress, and sensitivity to treatment with neuraminidase.[255, 261, 275, 276] In addition to the presence of α-2,3–linked terminal sialic acid residues, fucose residues located at specific linkage sites are critical for selectin binding. Under some conditions, all three selectins can bind to carbohydrate structures containing sialylated Lewis X antigen (sLe^x, CD15s) or its isomer, sialyl Lewis A (sLe^a) (Fig. 15–4).[34, 36, 160, 194, 310, 392, 553] On neutrophils and B lymphocytes, these sialylated structures may be carried on CD65, CD66, or additional surface molecules.[263, 405, 500] This is where the similarities end, because a number of important differences exist among ligands for selectins. For example, ligands for P-selectin are protease sensitive and endo–β-galactosidase resistant, whereas E-selectin ligands tend to be protease resistant and endo–β-galactosidase sensitive.[64, 274, 400, 571] This raises the possibility that ligands for P-selectin are sLe^x-containing glycoproteins and ligands for E-selectin are extended-chain forms of sLe^x, such as sialyl-dimeric Le^x (see Fig. 15–4), expressed as protease-resistant glycoproteins and/or glycolipids. For P-selectin, specific glycoprotein ligands have been discovered[340, 342, 372, 438, 590]; one has been named PSGL-1 (P-selectin glycoprotein ligand-1). The search for E-selectin ligands has also revealed several possible structures. Two extended-chain glycoprotein ligands for E-selectin on

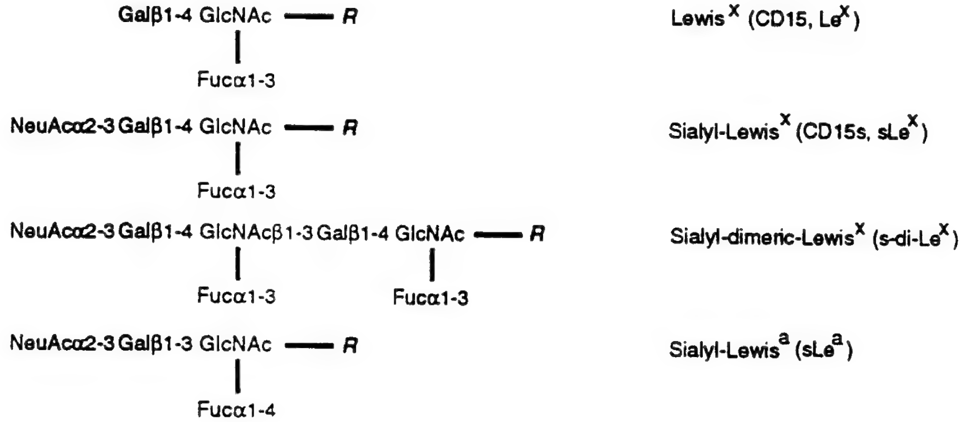

FIGURE 15–4. Chemical structures of several carbohydrate ligands for selectins. Gal = galactose; GlcNAc = *N*-acetyl glucosamine; NeuAc = neuraminic (sialic) acid; Fuc = fucose. R represents additional glycolipid and/or glycoprotein structures to which these terminal sugars may be attached. Note that sLe^x and sLe^a differ only in their galactose and fucose linkages.

the human monocytic cell line U937 were isolated.[390] Using mouse leukocytes, a variant of the fibroblast growth factor receptor was identified as an E-selectin ligand termed ESL-1 (E-selectin ligand-1).[442] Other studies suggest the presence of glycolipid ligands (e.g., sulfated structures such as galactosylceramides) for selectins on leukocytes.[23, 180, 502, 525] For subsets of memory skin-homing lymphocytes, additional sialylated molecules recognized by monoclonal antibodies HECA-452 (the cutaneous lymphocyte antigen) and 2F3 appear to mediate binding to E-selectin but not P-selectin.[38, 379, 394–396, 427] It has also been suggested that carbohydrate structures on L-selectin can interact with E-selectin and P-selectin.[397, 540] For L-selectin, ligands include the sulfated murine molecule glycosylated cell adhesion molecule-1 (GlyCAM-1),[220, 277] CD34,[illegible] and mucosal addressin cell adhesion molecule-1 (MAdCAM-1),[75] each belonging to the sialomucin family of adhesion molecules[405]; heparin[369, 373]; a sulfoglucuronyl glycolipid ligand that also binds P-selectin but not E-selectin[365]; and an antigen on high endothelial venules from human lymph nodes recognized by monoclonal antibody 2H5.[444]

Studies have begun to define the pathways responsible for synthesis of carbohydrate counterligands for selectins such as sLe^x. Biosynthesis of sLe^x results from the sequential activity of sialyltransferases and fucosyltransferases, particularly α-1,3 fucosyltransferases (Fuc-T) on α-2,3–sialylated lactosamine-type oligosaccharides.[261, 299] To date, five forms of α-1,3 fucosyltransferases have been cloned.[173, 256, 300, 575] One in particular, Fuc-TVII, appears to be especially important for leukocyte synthesis of sLe^x.[364, 443] Interestingly, transfection of this enzyme into cell lines that lacked sLe^x expression resulted in both sLe^x expression and E-selectin binding, although L-selectin binding was not observed.[501] This further highlights the subtle differences in carbohydrate binding specificities for different selectins. In umbilical vein endothelial cells, two α-2,3 sialyltransferases and four α-1,3 fucosyltransferases have been detected. TNF treatment results in increases in fucosyltransferase activity, sLe^x expression, and mRNA levels for Fuc-TVI in association with increased expression of sLe^x.[312] Clearly, additional investigation is needed to identify the specific glycosylated ligands for selectins on normal human cells and to characterize more accurately their affinities for different selectin ligands. Greater understanding is needed of whether there are differences in expression of these enzymes among leukocyte or endothelial subtypes, the regulation of expression and activity of these enzymes, and their specificity for carbohydrates on glycolipid versus glycoprotein substrates.

OTHER ADHESION MOLECULES

Several other adhesion molecules on endothelial cells and/or leukocytes have been identified; some may function during leukocyte recruitment responses. For example, vascular adhesion protein-1 (VAP-1), a 90-kD lymphocyte ligand, has been identified in synovial, mucosal, and peripheral lymph node endothelium and at sites of inflammatory disorders but not on unstimulated or activated umbilical vein endothelium.[439, 440] A similar molecule is L-VAP-2 (lymphocyte-vascular adhesion protein-2), a 70-kD structure constitutively expressed on umbilical vein endothelial cells and some lymphocytes; antibody-blocking studies suggest that it also functions as a lymphocyte ligand.[4] A pulmonary-specific endothelial cell adhesion molecule that participates in metastasis of tumors to the lung has been identified in mice (Lu-ECAM-1),[591, 592] but its counterpart in humans has not been identified. Another molecule, CD44 (formerly Hermes antigen, H-CAM or pgp-1), is found at high levels on most leukocytes, endothelial cells, epithelial cells, and other cell types.[286] Many splice variant forms of differing molecular weights have been identified (85–160 kD, with 90 kD most predominant). This family has been implicated as adhesion molecules for peripheral lymph nodes, hyaluronic acid, and T-cell signaling.[286] CD44 has also been shown to mediate interactions between lymphocytes and airway smooth muscle cells, inducing growth of the latter cell type.[281] The roles of these and other adhesion molecules in allergic inflammation remain to be determined.

PHYSIOLOGY OF CELL ADHESION: A STEPWISE PARADIGM OF CELL MARGINATION, ROLLING, ADHESION, AND TRANSENDOTHELIAL MIGRATION

A sequence of steps is likely involved during the emigration of leukocytes from the intravascular compartment into tissue sites.[3, 10, 83, 178, 485] Under the influence of blood flow, which causes shear forces to be applied to circulating leuko-

cytes, cells undergo a reversible process during which they roll or reversibly attach to the endothelium. Studies employing assays of adhesion under rotational conditions,[253, 483] using flow chambers in vitro,[1, 28, 84, 228, 279, 280, 305, 306, 581] or employing intravital video microscopy in vivo, using tissues such as rat mesentery,[134, 260, 289, 290, 382, 480, 486, 520, 540–542, 593] suggest that these tethering adhesive interactions are mediated primarily by interactions between carbohydrates and their selectin counterligands. However, other studies suggest that the integrin VLA-4 can also participate in cell rolling and arrest for cells expressing this molecule, such as eosinophils, lymphocytes, and monocytes.[12, 228, 305, 306, 486]

The next step requires leukocyte activation, perhaps as a result of their exposure to leukocyte-activating factors produced by and/or displayed on endothelial cells, such as PAF,[298, 595] chemokines, such as IL-8 for neutrophils,[215] and MIP-1β for T cells.[507, 508] Alternatively, contact of leukocytes with endothelial adhesion molecules may activate the cells directly.[294] Associated with these events are increases in both avidity and expression of integrins on the leukocyte surface.[128, 307, 481] Leukocyte rolling and activation are followed by firm leukocyte-endothelial adhesion, mediated by β1 and β2 integrins on leukocytes and VCAM-1, ICAM-1, and E-selectin on cytokine-activated endothelium.[46, 47, 55, 57, 61, 131, 132, 177, 190, 191, 251, 266, 270, 347, 383, 450, 451, 522, 550, 572] Subsequent transendothelial migration (diapedesis), during which the leukocytes emigrate between endothelial cells and penetrate the basement membrane[illegible] to enter the extravascular space, is mediated by PECAM-1,[124, 351, 353, 536] although integrins, selectins, and their ligands may also participate.[25, 100, 143, 161, 191, 304, 327, 344, 383] Cytokines, chemokines, and other chemotactic factors, by directly activating leukocyte migration responses, can potentiate the process of adhesion and transendothelial migration.[30, 144, 147, 215, 345, 478, 586, 587]

Further support for this paradigm is, in general, provided by studies of patients with genetic defects in human leukocyte β2 integrins (leukocyte adhesion deficiency type 1)[15, 16, 197, 539] and defects in generation of selectin ligands (leukocyte adhesion deficiency type 2)[154, 197, 409, 539] and studies of adhesion molecule knock-out mice (Table 15–4).[20, 77, 189, 267, 322, 477, 513, 577, 579] However, one important exception to this paradigm is seen in the immune response to bacterial infections of the lung. It appears that neutrophil recruitment into the lung is unaffected in P-selectin/ICAM-1 dual knock-out mice,[77] in mice treated with CD18 antibodies,[133] or in patients with leukocyte adhesion deficiency type 1,[15] suggesting the presence of a recruitment pathway that is independent of CD18, ICAM-1, and P-selectin. Whether this pathway is unique to the lung is not known.

Given the significant redundancy in adhesion molecule function, it seems almost certain that preferential recruitment of a given cell type would be the net result of many separate events rather than the effect of a unique cell-specific adhesion molecule pathway for each cell type. This paradigm would predict that a specific leukocyte infiltrate results from a series of relatively selective recruitment events in which overlapping cell adhesion mechanisms and chemotactic factors function in concert. For many cell types, evidence is rapidly accumulating

TABLE 15–4
MANIFESTATIONS OF ADHESION MOLECULE DEFICIENCY STATES IN HUMANS AND KNOCK-OUT MICE

Adhesion Molecule Deficiency	Consequences
Human	
Leukocyte Adhesion Deficiency type I (CD18 deficiency)[15, 16, 197, 539]	Blood neutrophilia with tissue neutropenia, delayed umbilical cord separation, recurrent soft tissue infections, impaired pus formation, and wound healing; pulmonary infections are not usually seen, and eosinophils and mononuclear cells, unlike neutrophils, can be found at sites of soft tissue infections; in vitro or ex vivo: reduced or absent neutrophil adhesion, transendothelial migration, and chemotactic responses are seen, although rolling adhesion is normal
Leukocyte Adhesion Deficiency Type II (Fucose Metabolism Defect)[154, 409, 539]	Severe mental retardation, short stature, distinctive facial appearance, Bombay (hh) blood phenotype, impaired pus formation, recurrent pneumonia, periodontitis, otitis, and cellulitis; neutrophil studies in vitro or ex vivo: reduced or absent sLe^x expression, reduced rolling adhesion, normal firm adhesion and migration in response to chemotactic stimulation
Mouse	
ICAM-1 Knock-out[77, 477, 579]	Impaired leukocyte recruitment to inflamed peritoneum and to sites of contact sensitivity, neutrophilia (~4–5 × normal), lymphocytosis (~2 × normal), improved resistance to LPS-induced shock
CD 18 Hypomorphic Mutation[577]	Impaired leukocyte recruitment to inflamed peritoneum and to sites of contact sensitivity, neutrophilia (~2–3 × normal), lymphocytosis (~1.5 × normal)
L-Selectin Knock-out[20, 511]	Markedly reduced leukocyte rolling and recruitment to inflamed peritoneum and to sites of contact sensitivity, improved resistance to LPS-induced shock, small lymph nodes, splenomegaly, normal antibody production
E-Selectin Knock-out[267]	Normal; profound impairment of PMN recruitment after infusion of P-selectin mAb
P-Selectin Knock-out[77, 322]	Absent leukocyte rolling, neutrophilia (~2–3 × normal), delayed PMN recruitment to inflamed peritoneum
VCAM-1 Knock-out[189]	Embryonic lethal
Dual ICAM-1/P-Selectin Knock-out[77]	Complete blockade of PMN recruitment during bacterial-induced peritonitis; no inhibition of PMN recruitment during bacterial-induced pneumonitis; blood leukocyte counts similar to ICAM-1 knock-outs

in support of this hypothesis. For example, both in vitro and in vivo studies have identified a number of adhesion-related pathways that may be critical during allergic inflammation.[29, 53, 56, 62, 65, 67, 88, 265, 292, 335, 336, 415, 447, 449, 556, 557]

EOSINOPHIL, BASOPHIL, AND MAST CELL INTERACTIONS VIA SELECTINS, INTEGRINS, AND THEIR COUNTERLIGANDS

In examining mechanisms of allergic inflammation, one useful approach has been to identify processes that activate or mediate eosinophil, basophil, and/or mast cell adhesion responses but not for other leukocyte types (e.g., neutrophils). Such pathways would, in theory, have a much higher likelihood to be of relevance to cellular recruitment responses associated with allergic diseases. For example, in comparing adhesion of eosinophils and neutrophils, it was demonstrated that both cell types can bind to cytokine-activated endothelium under rotational conditions in an L-selectin–dependent manner, although the ability of neutrophils to adhere was much greater than that of eosinophils.[253] However, one L-selectin antibody, LAM 1-11, had an unexpected activity in that it inhibited eosinophil but not neutrophil adhesion under these conditions, raising the possibility that eosinophils utilize an epitope on the L-selectin molecule not used by neutrophils.[253] Basophils also express L-selectin, but no information is available on its function for this cell type. Basophils have been shown to shed this molecule upon activation in vitro or in vivo, although the shedding is less complete compared with other granulocytes.[63, 169]

Eosinophils have been shown to bind at least as well as neutrophils to P-selectin immobilized on plastic surfaces,[531, 571] when expressed on the surface of activated platelets,[244] or in tissue sections from nasal polyps.[504] However, the ability of several enzymes, including proteases, to reduce neutrophil binding was greater than that for eosinophils, suggesting that there may be subtle differences in the level of expression or biochemical composition of P-selectin ligands.[571] Unlike eosinophil adhesion to most other ligands, eosinophils failed to spread on P-selectin and exhibited a reduced capacity to degranulate or produce superoxide anion, suggesting that attachment to P-selectin inhibited eosinophil function.[531] Whether similar functional interactions occur between basophils and P-selectin is not known.

Studies using recombinant E-selectin immobilized on plastic plates confirmed that eosinophils and basophils, like neutrophils, are capable of binding to E-selectin.[64, 66] All three cell types adhered to E-selectin, and their adhesion was dependent on leukocyte surface expression of sialic acid, since neuraminidase treatment and removal of sialic acid abolished essentially all adhesive activity.[64, 66] Interestingly, basophils bound best to E-selectin, followed by neutrophils and eosinophils. This relative rank order of binding efficiency was not directly related to the quantity of sLe^x on the cell surface. Flow cytometric studies using antibodies specific for sLe^x, as well as an extended-chain form of sLe^x (sialyl-dimeric Le^x), revealed that neutrophils had the greatest amount of sLe^x, whereas all three cells had comparable levels of sialyl-dimeric Le^x.[64, 66] That the extended-chain form of sLe^x may be responsible for adhesion to E-selectin was suggested by experiments utilizing endo–β-galactosidase. This enzyme removes the sialyl-dimeric Le^x and almost completely inhibited binding of all three cell types.[64, 66] Thus in spite of the fact that the bulk of sLe^x remained on the surface of neutrophils and basophils after treatment with endo–β-galactosidase, adhesion was dramatically impaired. These same experiments revealed that sialyl-dimeric Le^x is the important and predominant form of sLe^x on the eosinophil surface. Similar conclusions were reached in a study of NK cells.[400] Sialylated, extended-chain glycoprotein ligands for E-selectin have been identified on U937 cells, a human monocyte-like cell line.[390]

Among the earliest studies of cell adhesion were those in which cytokine-activated monolayers of cultured umbilical vein endothelial cells were treated for several hours with IL-1, TNF, or other stimuli, resulting in enhanced adhesion for neutrophils, eosinophils, and basophils.[47, 57, 61, 139, 270, 450] Antibodies to CD18, ICAM-1, and E-selectin inhibited adherence of all three leukocyte types.[57, 61, 266, 270] In contrast, anti–VCAM-1 antibody was extremely effective in inhibiting eosinophil adherence but had no effect on neutrophil adherence, suggesting that eosinophils, unlike neutrophils, recognize VCAM-1.[57] In these studies, basophil adherence was also demonstrated to be partly mediated through VCAM-1, although the inhibitory effect seen with anti–VCAM-1 antibody was less impressive. Furthermore, antibodies to VLA-4 inhibited eosinophil and basophil, but not neutrophil, adhesion to IL-1–stimulated endothelium, corresponding to the expression pattern of the VLA-4 counterligand.[57, 66, 131, 550, 572] The ability of eosinophils and basophils to adhere to VCAM-1 was confirmed by showing that these cells could adhere to an immobilized recombinant form of VCAM-1 and in experiments in which the adhesion was inhibited using VCAM-1 and VLA-4 antibodies.[66, 451, 572]

The finding that VCAM-1/VLA-4–mediated adhesion was different among eosinophils and basophils compared with neutrophils raised the possibility that specific induction of VCAM-1 expression on endothelial cells might promote eosinophil and basophil adherence but not neutrophil adherence. Previous studies suggested that the cytokine IL-4 was capable of selectively inducing VCAM-1 expression in endothelial cells.[522] Incubation of endothelial cells with IL-4 did not influence neutrophil adherence but did induce eosinophil and basophil adherence that was inhibited by more than 70% with either anti–VCAM-1 or anti–VLA-4 antibodies.[451] Similar results have been obtained with IL-13, a cytokine that shares many biologic activities with IL-4.[55] These in vitro findings were consistent with several in vivo murine studies: intraperitoneal or intradermal injection with IL-4 resulted in an eosinophil-rich infiltrate[346]; IL-4 transgenic mice developed tissue eosinophilia and an allergic-like syndrome[515]; mice inoculated with an IL-4 transfected tumor cell line developed local eosinophilia at the tumor site[516]; and anti–IL-4 reduced antigen-induced expression of VCAM-1 in mouse trachea and eosinophil recruitment to the lung.[301, 362] Therefore, IL-4, by selectively promoting VCAM-1 expression, might contribute to the preferential recruitment of eosinophils (and basophils) compared with neutrophils seen during certain inflammatory responses.

The discovery that eosinophils and basophils, but not neutrophils, express α4β7,[66, 524] a molecule that recognizes both VCAM-1 and MAdCAM-1,[17, 42, 153] suggests that this integrin may also play a role in preferential recruitment

responses. However, α4 integrins are expressed on other cell types, including lymphocytes and monocytes.[204] There also are situations in vivo in which acute or chronic eosinophil accumulation occurs without significant endothelial VCAM-1 expression[266, 338, 479] or under conditions in which VCAM-1 is expressed at relatively high levels but little or no eosinophil accumulation is seen.[72, 184, 185] Thus it seems unlikely that the VCAM-1/VLA-4 adhesion pathway is solely responsible for selective eosinophil and basophil recruitment.

Several in vitro studies have begun to analyze the molecular mechanisms regulating eosinophil transendothelial migration. Treatment of endothelial monolayers with IL-1 or TNF increased eosinophil transendothelial migration.[143, 345] Eosinophil transendothelial migration through IL-1–treated endothelium was almost completely inhibited by antibodies to CD18, but CD29 antibody had little or no effect.[143] Antibodies directed against the LFA-1 binding site on ICAM-1 were moderately effective in inhibiting transmigration, although a combination of VCAM-1, ICAM-1, and E-selectin antibodies was more effective than ICAM-1 antibody alone.[143] Cytokines such as GM-CSF or IL-5 will markedly potentiate eosinophil transendothelial migration across unstimulated or cytokine-activated endothelial cell monolayers.[144, 346] In another study, an antibody that activates, rather than inhibits, β1 integrin function dramatically inhibited eosinophil chemotaxis and transendothelial migration, presumably by enhancing adhesion and essentially immobilizing these cells.[264] These data support the hypothesis that transendothelial migration of eosinophils involves the function and expression of adhesion molecules on both the leukocyte and the endothelium and suggest that the mechanisms regulating leukocyte adhesion may differ from those mediating transmigration. Thus far, the role of PECAM-1 in eosinophil transmigration remains unknown, and mechanisms of basophil transendothelial migration have not been examined.

Several stimuli, including cytokines, possess the ability to selectively enhance eosinophil or basophil adhesion-related responses. For example, exposure of eosinophils to IL-3, IL-5, or GM-CSF will promote adhesion molecule function, induce L-selectin shedding and CD11b up-regulation, and facilitate chemoattractant-induced adhesion responses and transendothelial migration.[50, 144, 198, 367, 527, 549, 554, 558, 559] Exposure to the chemokine RANTES, a potent and selective eosinophil activator and chemoattractant in vitro[5, 235, 428, 582] and in vivo,[31, 331] causes eosinophil transendothelial migration.[147] The effects of RANTES on eosinophils were synergistic with IL-5, and both anti-CD18 and anti–VLA-4 antibodies were needed to effectively inhibit RANTES-induced transmigration across IL-1–activated endothelium.[147] Interestingly, eosinophils purified from late-phase reaction bronchoalveolar lavage (BAL) fluids display a similarly potentiated transendothelial migration response.[148] Furthermore, eosinophil-activating cytokines have been detected at sites of allergic inflammation,[140, 143, 241, 317, 343, 380, 381] and both epithelial and endothelial cells have been shown to produce RANTES.[32, 493–495]

Taken together, these data suggest that RANTES and perhaps other C-C chemokines with eosinophil chemotactic activity,[113a, 228a, 376a] especially in the presence of priming cytokines, may play important roles during eosinophil transmigration in vivo. For basophils, IgE-dependent degranulation or treatment with IL-3 will enhance adhesion to endothelial cells in a β2 integrin–dependent manner.[58, 59] Whereas IL-3 and IL-5 are basophil chemoattractants[510]; the C-C chemokines are more potent and have little or no effect on neutrophils.[5a, 49a, 113a, 564a] In each instance, these treatments have little or no effect on neutrophils, and adhesion is mediated primarily through β2 integrins. Preliminary data suggest that β1 integrins on eosinophils exist in a state of partial activation and can be maximally activated for adhesion to VCAM-1 after exposure to certain divalent cations (e.g., Mn^{2+}) or integrin-activating antibodies; these are conditions that do not affect the total cell surface expression of β1 integrins.[320, 574] In contrast, IL-5 prevents cation-induced β1 integrin activation, as did the tyrosine kinase inhibitor tyrphostin.[321, 574] These data suggest that cytokines can cause functional activation of certain adhesion pathways while down-regulating expression and function of other adhesion pathways; the balance determines whether cell adhesion or migration will occur. This occurrence, in fact, has been reported in hematopoietic cell lines.[258]

Once leukocytes enter the extravascular space, migration through the tissue is dependent on their ability to bind to extracellular matrix proteins. Interactions with matrix proteins also appear to be important in mast cell localization within tissues. Since most of the receptors for matrix proteins belong to the β1 integrin family, eosinophils, basophils, and mast cells were examined for their expression of these surface structures.[65, 145, 170, 188, 532, 533] Eosinophils can bind to fibronectin via α4 integrins,[18, 366] although activation of the integrin may be required for binding.[320, 321] Eosinophils can also bind to laminin via α6 integrins.[170] Interestingly, adhesion to fibronectin was shown to activate eosinophil production of superoxide anion,[136] degranulation responses,[366] leukotriene release,[18] and production of IL-3 and GM-CSF, which augmented eosinophil survival in an autocrine fashion.[19] Eosinophil degranulation, aggregation, and cytokine production in response to several stimuli or on various surfaces, including matrix proteins, can be regulated via β2 integrins.[149, 212, 514]

Studies examining expression and function of β1 integrins in human basophils revealed expression of α4 and α5 integrins that are capable of mediating adhesion to VCAM-1 and fibronectin.[65, 66, 333, 532] In addition to α4 and α5 integrins, mast cells also express α3 integrins; the latter mediate adhesion and migration to laminin, whereas all three are capable of mediating fibronectin binding.[108, 116, 518, 519] Mast cell interactions with laminin may be important in tissue localization in vivo.[551] Basophils from asthmatic (but not normal) donors will release histamine upon antibody cross-linking of β1 integrins, whereas IgE-dependent basophil and mast cell mediator release is inhibited.[174, 175, 278] In other studies using rat, mouse, and cultured human mast cells, interactions with fibronectin appeared to enhance IgE-dependent histamine and cytokine release.[333, 411] Adhesion mediated via integrins, or cross-linking of cell surface integrins, can therefore affect a wide range of biologic activities on eosinophils, basophils, and mast cells.

EXPRESSION AND FUNCTION OF ADHESION MOLECULES IN VIVO DURING ALLERGIC AND OTHER INFLAMMATORY RESPONSES IN THE SKIN AND AIRWAYS

One of the first pieces of evidence suggesting the existence of different adhesion-related mechanisms for cell recruitment

among granulocyte subtypes was found in patients with leukocyte adhesion deficiency type I, in which eosinophils and mononuclear leukocytes are able to accumulate at sites of infection even though neutrophils are not.[15] These findings suggested that eosinophils, unlike neutrophils, possessed β2 integrin–independent mechanisms for recruitment into tissues probably due, at least in part, to α4 integrins.

The potential role of adhesion molecules in allergic diseases has been the topic of several reviews.[29, 62, 88, 336, 556, 557] Investigators studying this question have taken a number of approaches. One approach has been to attempt to find evidence of endothelial activation at sites of allergic inflammation. The expression of endothelial adhesion molecules has been examined in the skin, nose, and airways following experimental allergen challenge and in allergic and other eosinophilic diseases. Studies using immunohistochemical techniques have demonstrated that intradermal injection of allergic individuals with allergen activates the vascular endothelium to express E-selectin and VCAM-1, and to increase its expression of ICAM-1, in an IgE-dependent manner.[56, 266, 287, 448] Increased expression of VCAM-1 was also observed 24 hours after intranasal allergen challenge, and the numbers of eosinophils infiltrating the tissues correlated, albeit weakly, with the extent and intensity of VCAM-1 staining.[282] Endobronchial allergen challenge resulted in increases in endothelial VCAM-1 staining and epithelial ICAM-1 staining, with a significant correlation between these parameters and eosinophil influx. Allergen challenge of the eye induced ICAM-1 expression on conjunctival epithelium.[102]

There is indirect evidence that endothelial activation also occurs within the human airway following endoscopic intrabronchial allergen challenge, since increased levels of soluble forms of E-selectin, ICAM-1, and VCAM-1 are observed in BAL fluids[169, 506, 589]; in one of these studies, there was a correlation with eosinophil influx and levels of both IL-4 and IL-5.[589] One study found an increase in serum levels of soluble ICAM-1 and E-selectin, but not VCAM-1, in patients admitted for exacerbations of asthma.[337] In nonhuman primates, allergen inhalation resulted in E-selectin expression on the airway vascular endothelium within 6 hours.[186] The pattern of endothelial activation seen during allergic inflammation in vivo suggests that endothelial cells are being exposed to cytokines such as IL-1, TNF, and/or IL-4. Possible cellular sources for IgE-dependent release of these endothelial-activating cytokines are mast cells and basophils. In vitro studies have demonstrated that IgE-dependent stimulation of mast cells results in increased mRNA levels and secretion of a variety of endothelial-activating cytokines, including TNF, IL-4, and IL-13.[59, 71, 78, 140, 163, 176, 552] Basophil production of more impressive quantities of IL-4 has also been clearly demonstrated.[76, 309, 350, 454] Several studies have found evidence that these cytokines may be generated during allergic reactions. For example, IL-1 and IL-4 are released in vivo during experimental allergic reactions,[52, 589] and mRNA has been detected at allergic inflammatory sites for TNF,[585] IL-4,[71, 140, 241, 418, 419, 584] and IL-13.[214]

Especially convincing is the observation that E-selectin expression induced by allergen injection in human skin can be inhibited if the site is immediately biopsied and placed into culture with a mixture of antibodies that neutralize IL-1 and TNF.[287] Eosinophil influx has been observed following injection of IL-1 into the skin of rats[442] or following injection of IL-4 into the skin or peritoneum of mice[346] but not in baboons, despite induction of VCAM-1 expression.[72] In guinea pigs, an IL-1 antagonist inhibited airway hyperreactivity and the development of pulmonary eosinophilia.[561] However, studies in which biopsies were performed after IL-1 or TNF was injected into the skin of humans revealed an intense leukocytic infiltration devoid of eosinophils.[184, 185] Furthermore, it was demonstrated that an IL-1 antagonist could effectively inhibit the human cutaneous late-phase response; yet no significant inhibitory effect on cell influx was seen histologically. It therefore seems likely that other factors, besides nonspecific endothelial activation alone, are required for eosinophil recruitment responses to occur in vivo.[205]

Changes in the expression of cell surface adhesion molecules occur on eosinophils and basophils during their movement from the circulation into tissues, implying an involvement during experimental allergic inflammation. Comparisons of levels of adhesion molecules on granulocytes recovered from blood and either sputum, bronchoalveolar lavage, or nasal lavage post antigen challenge revealed increased expression of CD11b,[29, 169, 196, 259, 457] diminished levels of L-selectin,[29, 169, 328] and little or no change in expression of LFA-1, VLA-4, or sialyl-dimeric Le^x (unpublished observations).[169, 259, 457] Similar phenotypic changes have been observed after eosinophil transendothelial migration in vitro (see Fig. 15–2).[143, 548] Although these data demonstrate dynamic changes in eosinophil and basophil adhesion molecule expression during inflammation, these changes also occur on neutrophils and have been observed on cells obtained from other inflammatory reactions or on those that spontaneously migrate to these sites.[232, 248] Therefore, these events cannot, by themselves, account for selectivity in cell recruitment but instead probably represent a common consequence of cell recruitment.

Several experiments implicating cell adhesion molecules in the pathophysiology of allergic rhinitis and asthma have now appeared. When bronchial mucosal biopsies were obtained from normal subjects and those with mild, stable allergic asthma, immunohistochemical analyses revealed similar levels of endothelial expression of ICAM-1 and E-selectin (VCAM-1 was not studied), despite an increased number of eosinophils in the mucosa of the asthmatic subjects.[338] After 6 weeks of treatment with inhaled budesonide, tissue eosinophilia in the asthmatic subjects was reduced; yet no significant change in the pattern of ICAM-1 and E-selectin expression was observed. A subsequent study compared endothelial adhesion molecule expression in airway biopsies from subjects with allergic and nonallergic asthma as well as normal controls.[33] Constitutive expression of ICAM-1, VCAM-1, and E-selectin was observed in all groups. Endothelial staining for ICAM-1 and E-selectin, but not VCAM-1, was significantly increased in the nonallergic asthmatic group only, whereas epithelial staining for ICAM-1 was increased in both groups of asthmatic subjects.

This is in contrast with another study in which more symptomatic patients with asthma underwent bronchoscopy and biopsy. In this study, strong endothelial staining for VCAM-1, as well as ICAM-1, was observed.[378] Studies of nasal airway tissue from subjects with perennial allergic rhinitis found increased expression of ICAM-1 and VCAM-1, but not E-selectin, compared with tissue from nonallergic

TABLE 15–5
ANTIADHESION TREATMENTS TESTED IN ANIMAL MODELS OF ALLERGIC OR OTHER INFLAMMATORY DISEASES OF THE AIRWAYS OR SKIN

Inflammatory Model	Animal	Treatment*
Airways		
Antigen-induced eosinophil recruitment and airway responsiveness	Monkey	ICAM-1 mAb (iv or inhaled)[568, 569]
Antigen-induced eosinophil recruitment and allergic late-phase responses	Rabbit	VLA-4 mAb, CS-1 peptide[330]
	Sheep	VLA-4 mAb (no efffect on eosinophils)[2]
	Guinea pig	CD18 mAb[332] VLA-4 mAb[408]
Antigen-induced T-cell and eosinophil recruitment to trachea	Rat	VLA-4, VCAM-1 mAb[362] ICAM-1, CD11a mAb (no effect on eosinophils)[362]
Antigen-induced airway responses and leukocyte recruitment	Rat	CD11a, CD11b, VLA-4 mAb (blocked airway responses but not cell recruitment)[413]
Antigen-induced neutrophil recruitment and allergic late-phase responses	Monkey	E-selectin mAb[186] ICAM-1 mAb (no effect)[186]
Chronic eosinophilic airway inflammation	Monkey	ICAM-1 mAb (no effect)[187]
Cobra venom factor-induced, PMN-mediated lung inflammation	Rat	P-selectin mAb[357] sLex and derivatives[356] L-selectin F(ab')2 mAb[355] P-selectin chimera[360] L-selectin chimera[360] E-selectin chimera (no effect)[360]
IgG immune complexes, PMN-mediated lung inflammation	Rat	E-selectin mAb[359] sLex and derivatives[354] PECAM-1 mAb[536] L-selectin mAb[355] L-selectin mAb (iv; not effective it)[358] ICAM-1 mAb (iv or it)[358] CD11a mAb[361] CD11a mAb (iv; not effective it)[358] CD11b mAb (not effective)[361] CD11b mAb (it; not effective iv)[358]
IgA immune complexes, macrophage-mediated lung inflammation	Rat	ICAM-1 mAb[361] CD11a mAb[361] CD11b mAb[361] VLA-4 mAb[361] E-selectin mAb (no effect)[359] sLex and derivatives (no effect)[354] L-selectin F(ab')2 mAb (no effect)[355]
Bacterial-induced subcutaneous PMN migration	Rabbit	CD18 (no effect)[113, 202]
GM-CSF–induced intrapulmonary PMN sequestration	Monkey	CD18 mAb (small effect)[587] CD11b mAb (no effect)[587]
Actinomycetes-induced pneumonitis and fibrosis	Mouse	CD11a[126]
TNF-induced PMN sequestration, pulmonary edema	Guinea pig	ICAM-1, CD11b, CD18 mAb[293]
Skin		
PAF, LTB, and C5a des arg–induced eosinophil influx	Guinea pig	VLA-4 mAb[565]
Delayed-type hypersensitivity	Rat	CD11a mAb[223] VLA-4 mAb[222] CD11a plus VLA-4 mAb[221]
	Mouse	VLA-4 mAb[98] CD11a, ICAM-1 mAb[445]
	Monkey	E-selectin, VCAM-1 mAb[471]
Bacterial-induced subcutaneous PMN migration	Rabbit	P-selectin (no effect)[461]
FMLP-induced PMN infiltration	Rabbit	CD11a, CD18[433] CD11b (no effect)[433]
TNF-induced PMN influx into transplanted human skin	SCID mouse	PECAM-1[536] E-selectin[583]

*Treatments were effective and given intravenously unless otherwise noted.
Abbreviations: mAb = monoclonal antibody; iv = intravenous; it = intratracheal; PMN = neutrophil.

controls.[334] Seasonal exposure to pollen was associated with a significant increase in nasal epithelial cell expression of ICAM-1 along with increased numbers of eosinophils, neutrophils, and metachromatic cells.[103] Further support for the potential role of VLA-4 VCAM-1–mediated eosinophil recruitment was provided by the demonstration of VCAM-1 staining of blood vessels, without E-selectin staining, in skin biopsies from patients with eosinophilic vasculitis[97] and significant VCAM-1 staining in nasal polyps, tissues in which extensive eosinophilia is seen.[32]

Ultimately, direct proof of adhesion molecule involvement in inflammation of allergic and other diseases will require studies employing specific adhesion molecule antagonists.[201, 529, 566, 570] Although no data have been reported for allergic diseases in humans, humanized antibodies to ICAM-1, CD18, VLA-4, and perhaps others are now available and have been or will be used in other types of clinical trials.[200, 240, 295, 475] These efforts have been motivated in large part by the success in animal studies. Blocking monoclonal antibodies have been infused in vivo in a variety of animal models of allergic and other inflammatory conditions of the lung and skin (Table 15–5).[2, 48, 126, 133, 186, 187, 202, 221–223, 293, 330, 332, 354–362, 408, 413, 433, 445, 461, 471, 536, 565, 568, 569, 583, 587] From several of these studies, however, it became evident that blockade of adhesion molecules might not prevent cell influx yet still have clinical benefit. Examples of this include studies in monkey and sheep models of asthma. In monkeys, antibodies against leukocyte CD11b were given systematically, and the antigen-induced rise in airway responsiveness was inhibited, even though the number of eosinophils recovered by bronchoalveolar lavage was unaffected.[567] Interestingly, eosinophil peroxidase activity in the bronchoalveolar lavage fluids was reduced by antibody treatment, suggesting an effect of the antibody on eosinophil activation and degranulation rather than on recruitment per se. A study using a sheep model also found efficacy of a VLA-4 antibody in preventing late-phase changes in airway function without any significant effect on leukocyte recruitment as assessed by bronchoalveolar lavage.[2] Thus antibodies to cell adhesion molecules may affect cell functions as well as trafficking. Many novel pharmaceutical approaches are being tried in an attempt to prevent cell recruitment responses, and results from additional studies in humans should be forthcoming.[201, 555, 570] Only then will we truly be able to functionally define the role of adhesion molecules and cytokines in human allergic diseases, including rhinitis and asthma.

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16 Chemotactic Molecules and Cellular Activation

NICHOLAS W. LUKACS PETER A. WARD

The movement of leukocytes from the vasculature, through the vessel wall, and into tissue to the site of inflammation is a complex, coordinated, multistep event. Chemotaxis of leukocytes is a central occurrence during immune responses that acts to localize cells to a site of infection or injury. Chemotaxis of leukocytes is initiated by increases in the expression of adhesion molecules on the vascular endothelial cells that allows localization and adherence of leukocyte populations to the area of the inflammatory response. These early adhesion events are induced by a number of early response mediators and cytokines, including histamine, C5a, tumor necrosis factor-alpha (TNF-α), and IL-1. The early response cytokines and mediators up-regulate selectin molecules (E and P) on the endothelial surface and slow the leukocytes from circulatory flow, a phenomenon known as rolling.

After leukocytes have been localized to the inflamed vascular wall, the cells firmly adhere to the endothelium by interaction with adhesion molecules (ICAM-1 and VCAM-1), which allow the spreading of the leukocytes along the endothelial surface.[34, 76] Once firmly adhered to the vascular endothelium, leukocytes can migrate into the tissue, following along chemotactic gradients that not only localize the cells to the site of inflammation but also appear to prime and

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CHAPTER 9

Cellular Adhesion in Inflammation

Bruce S. Bochner

One of the hallmarks of an inflammatory reaction is the localized accumulation of subsets of leukocytes within a tissue site. In the lung, for example, the preferential recruitment of neutrophils during bacterial pneumonia, eosinophils during an experimental allergic late-phase reaction, or T cells during hypersensitivity pneumonitis are but a few examples of the ability of tissue-resident cells, along with cells of the peripheral immune system, to orchestrate a wide range of inflammatory responses in which different patterns of cell influx are observed. Among the molecules that contribute to these selective recruitment responses are those that permit cell-cell and cell-substratum attachment. These structures, collectively referred to as *cell adhesion molecules*, are now known to be necessary for essentially every step in cell recruitment, including leukocyte-endothelial interactions (margination), diapedesis (transendothelial migration), directed movement through tissues (chemotaxis and haptotaxis), and, in the lung, transepithelial migration. More than 35 adhesion molecules have already been characterized molecularly and biochemically on human cells. These molecules are subdivided into families (selectins and their sialomucin counterligands, integrins, immunoglobulin-like structures, and others) based on shared structural characteristics and functions. Insight into their functions has been gained through a number of approaches. For example, peptide and antibody-based adhesion molecule antagonists have been developed, some of which are now being tested in vivo. More recently, a variety of adhesion molecule knockout mice have been created that display uniquely altered inflammatory responses. These and other studies have been crucial in expanding the understanding of the biologic importance and relative contributions of these molecules in a variety of immunologic responses.

The overall goal of this chapter is to summarize the structural and functional characteristics of cell adhesion molecule families. A description of key molecular and biochemical characteristics is provided, along with a discussion of their respective ligands. This is followed by a summary of the regulation of their surface expression and function, both in vitro and in vivo. Because of the expansive nature of the topic, discussion has been restricted to those cells and molecules most relevant to allergic inflammation. However, attempts have been made to reference additional publications that cover specific topics in greater depth. Several comprehensive texts[1-3] and reviews on adhesion-related topics[4-6] may also be of interest.

SELECTINS AND THEIR LIGANDS

The first family of adhesion molecules discussed is the selectin gene superfamily.[7-9] The only three known members, E-selectin, L-selectin, and P-selectin, are also referred to as CD62 followed by their respective first letters (CD62E, CD62L, and CD62P). E-selectin (115 kD, originally named endothelial-leukocyte adhesion molecule-1 [ELAM-1][10] is expressed exclusively on activated endothelium. P-selectin (150 kD, also referred to as GMP-140 or PADGEM[11]), the largest selectin, originally received its name because of its stimulus-dependent expression on platelets, but it is also rapidly and transiently expressed on endothelial cells. L-selectin is the smallest selectin (formerly TQ1, LECCAM-1, LECAM-1, Leu-8, or LAM-1, 75 kD on lymphocytes, 100 kD on granulocytes, and 110 kD on monocytes[4,12]) and gets its name because of expression restricted to leukocytes. Although selectins can mediate adhesion under static conditions,[13-15] it is now felt that the major function of selectins in vivo is to mediate leukocyte-endothelial tethering and rolling under forces of shear stress.[16-18] L-selectin also functions during lymphocyte trafficking to peripheral and mesenteric lymph nodes.

The structures of the selectins are shown schematically in Figure 9-1. Each consists of an N-terminal domain of 117 to 120 amino acids possessing calcium-dependent (C-type) lectin activity.[19] Proximal to this region is a 32 to 38 amino acid segment with homology to a domain initially discovered in epidermal growth factor (EGF), the EGF domain. Proximal to this are 2 to 9 domains, each about 60 amino acids long, whose sequences resemble those found in complement regulatory proteins.[9] These domains extend the molecule out from the cell surface, facilitating rolling function.[20] The extracellular portions of selectins are anchored to the cell surface by transmembrane and intracytoplasmic domains of 21 to 35 amino acids. Unlike the extracellular domains, which share significant homology (40% to 60% overall, 60% to 70% within the lectin and EGF domains), little homology exists among the transmembrane and intracytoplasmic domains.[21] The most critical portion of the selectin molecule for adhesion is the lectin domain, although the conformation of the adjacent EGF domain may influence binding.[9] For L-selectin, but not for E- or P-selectin, the endogenous intracytoplasmic portion of the molecule is required for adhesive function.[22,23] Soluble forms of all three selectins, as well as other adhesion molecules, can be detected in blood and other body fluids and may possess biologic activities.[24,25]

E-selectin is not present on the surface of resting endothelium. Expression of E-selectin is inducible within several hours in cultured endothelial cells or tissue explants after exposure to various stimuli, including interleukin-1 (IL-1), tumor necrosis factor (TNF), and lipopolysaccharide (LPS).[26-30] Expression can be potentiated by interferon-γ (IFN-γ)[31] and inhibited by transforming growth factor-β (TGF-β).[32] Once expressed, E-selectin functions as a ligand for leukocytes, including neutrophils,[27,28,33] monocytes,[27] eosinophils,[14,34-36] basophils,[15] and subsets of T lymphocytes bearing the cutaneous lymphocyte antigen (CLA, see below).[37] Molecular studies of the E-selectin promoter have revealed that transcription is under the control of several transcription factors, including NF-κB.[38] Surface expression of E-selectin in vitro is relatively transient, with levels approaching those at baseline by 24 hours.[29] This is because most of the E-selectin that is expressed is reinternalized and degraded, although a small proportion is shed.[39,40] E-selectin expression at sites of inflammation in vivo appears to be more prolonged,[41] perhaps because of differences in posttranscriptional stability among forms of E-selectin transcripts.[42]

Like E-selectin, P-selectin is not present on the luminal surface of resting endothelium. However, unlike E-selectin, P-selectin exists preformed within granules (the Weibel-Palade bodies) and is expressed within minutes after stimulation with agents such as histamine, thrombin, phorbol esters, peroxides, C5a, and leukotriene C_4.[43] In contrast, prolonged exposure to cytokines such as IL-3 or IL-4 leads to a gradual and sustained increase in expression.[44,45] P-selectin has been shown to be a ligand for many cell types, including neutrophils, eosinophils, monocytes, and some T lymphocytes.[43,46] Leukocyte interaction with P-selectin has been shown to alter cellular functions, including superoxide production, integrin-mediated phagocytosis, and production of cytokines and chemokines.[47-49] In contrast, leukocyte activation can reduce adhesion to P-selectin, in part by altering the topographic location of P-selectin ligands on the cell surface.[50]

The third and smallest member of the selectin family, L-selectin, is found exclusively on leukocytes. It was originally discovered as a peripheral lymph node homing receptor responsible for lymphocyte attachment to high endothelial venules found in lymph nodes.[51,52] It also functions as an adhesion molecule for nonlymphoid vascular endothelium under conditions of shear stress.[53-56] L-selectin is shed through activation of an endogenous proteolytic pathway that releases the molecule from a site close to the cell membrane.[57,58] This process is

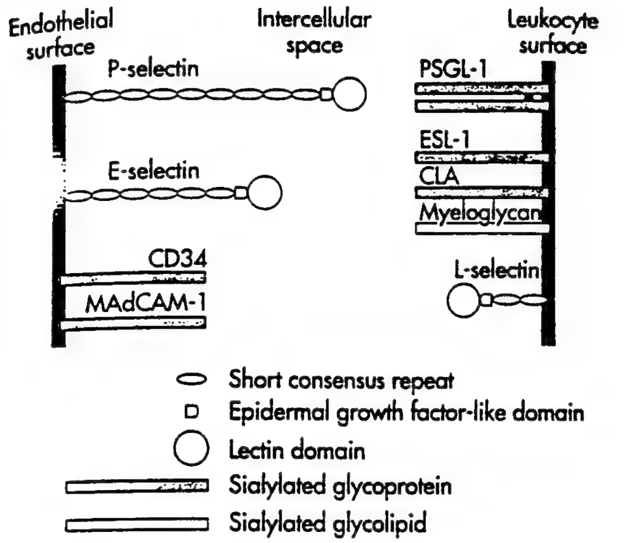

FIGURE 9-1 Basic structures of selectins on leukocytes and endothelium. Examples of extensively glycosylated, mucinlike counterligands for each selectin are also displayed. The transmembrane and intracytoplasmic domains for each of these structures are not shown. *PSGL-1*, P-selectin glycoprotein-1; *ESL-1* E-selectin ligand-1; *CLA*, cutaneous lymphocyteantigen; *MAdCAM-1*, mucosal addressin cell adhesion molecule-1.

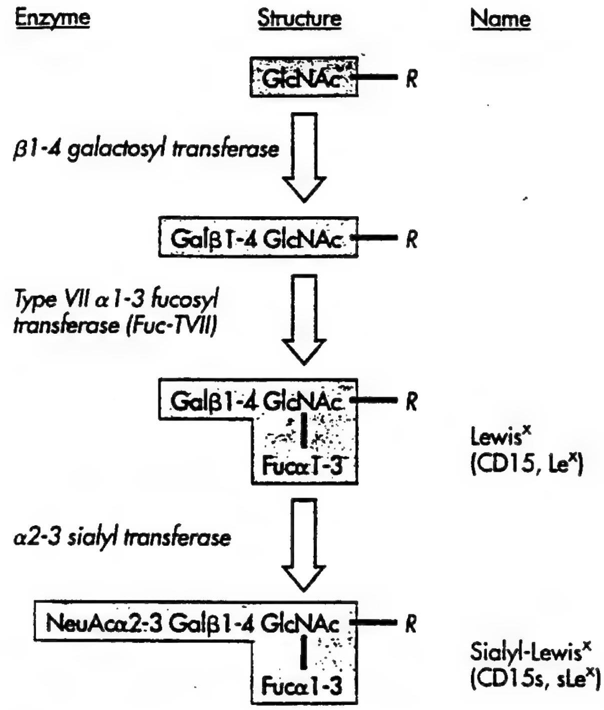

FIGURE 9-2 Structures and pathways for synthesis of carbohydrate ligands for selectins. *Gal*, Galactose; *GlcNAc*, N-acetyl glucosamine; *NeuAc*, neuraminic (sialic) acid; *Fuc*, fucose. *R* represents the core glycolipid or glycoprotein structure to which these terminal sugars may be attached.

activated during leukocyte activation by chemotactic factors, cytokines, and other stimuli[59] and conditions that interfere with L-selectin shedding alter cell rolling.[60]

A variety of carbohydrate-containing mucinlike ligands for selectins have been identified[8,61] (see Figure 9-1). Studies have focused on the core proteins or lipid structures on which the carbohydrates are expressed, the carbohydrates themselves, and the enzymatic pathways involved in glycosylation. Many of the characteristics of selectin-selectin ligand interactions are similar, including calcium dependence, function at low temperatures and under conditions of shear stress, and sensitivity to treatment with neuraminidase.[8,9] Although the tetrasaccharide sialyl Lewis^x (sLe^x), which contains α2,3-linked terminal sialic acid residues and α1,3-linked fucose (Figure 9-2; see below), can bind to all three selectins,[62-64] a number of important differences exist among ligands for selectins. For example, ligands for P-selectin on human leukocytes are protease sensitive and endo-β-galactosidase resistant, whereas E-selectin ligands are protease resistant and endo-β-galactosidase sensitive,[14,46,65] suggesting that the former is an sLe^x-containing glycoprotein and the latter may be an extended-chain, sLe^x-containing glycolipid. For P-selectin, at least one N-terminally sulfated, disulfide-linked homodimeric glycoprotein ligand, named *P-selectin glycoprotein ligand-1* (PGSL-1), has been identified and is widely expressed on leukocytes and other cells.[66-69] The search for E-selectin ligands has revealed several possible structures. On neutrophils and B lymphocytes, sialylated E-selectin ligands may be carried on CD65, CD66, L-selectin, or additional surface molecules.[37,70,71] Two extended-chain glycoprotein ligands for E-selectin on the human monocytic cell line U937 were recently described,[72] and other investigators, using mouse leukocytes, identified an E-selectin ligand that they termed *E-selectin ligand-1* (ESL-1), a variant of the fibroblast growth factor receptor.[73] Still other studies suggest the presence of glycolipid E-selectin ligands on leukocytes, such as galactosylceramides or polylactosaminolipids; the latter structures have been termed *myeloglycans*.[74-77] For subsets of memory (CD45RO$^+$), skin-homing lymphocytes, additional sialylated molecules recognized by monoclonal antibodies such as HECA-452 (CLA) appear to mediate binding to E-selectin but not to P-selectin.[37,51,78,79] For L-selectin, recently identified fucosylated, sialylated, sulfated ligands on endothelium include CD34,[80] mucosal addressing cell adhesion molecule-1 (MAdCAM-1)[17,81] and an as-yet-unidentified cytokine-inducible structure.[55,82] Another mucinlike structure identified in the mouse, glycosylated cell adhesion molecule-1 (GlyCAM-1),[83] appears to exist only in a soluble form, and its role in cell trafficking remains unclear. Each of these L-selectin ligands belong to the sialomucin family of adhesion molecules.[84]

Recent studies have begun to define the enzymatic pathways responsible for synthesis of carbohydrate counterligands for selectins such as sLe^x (see Figure 9-2). Biosynthesis of sLe^x results from the sequential activity of sialyltransferases and fucosyltransferases, in particular, α1,3 fucosyltransferases (Fuc-T) on α2,3-sialylated lactosamine-type oligosaccharides.[8,85] To date, five forms of α1,3 fucosyltransferases have been cloned,[8] but one in particular, Fuc-TVII, is especially important for leukocyte synthesis of sLe^x.[86] Support for the critical role of these glycosylation events in leukocyte trafficking in vivo has been provided in both animals and humans. The recent generation of a Fuc-TVII knockout mouse has revealed an extremely important role for this enzyme in the synthesis of ligands for all three selectins.[87] In addition, a rare genetic disease in which fucose metabolism is abnormal results in leukocyte adhesion deficiency disease type II (LAD type II), in which sLe^x synthesis and leukocyte rolling and recruitment responses are impaired.[88,89] Given the bewildering array of similarities and differences among selectins and their ligands, more studies are needed to distinguish among specific and nonspecific ligands for selectins on human cells.

INTEGRINS

The integrins have been grouped into a large family of structurally similar heterodimeric molecules with noncovalently associated α and β chains.[90,91] At least 16 α subunits and 8 β subunits have been identified that can combine to generate at least 23 different heterodimers (Table 9-1). Although it was initially felt that α and β subunit pairings were restricted according to the β subunits, different α subunits can associate with more than one β subunit.[91]

The structure of a typical integrin is shown schematically in Figure 9-3. The α and β subunits range in size from 120 to 210 kD and 90 to 110 kD, respectively, and in general there is more homology among β subunits than among α subunits. Within the extracellular portions of α subunits are three or four domains, each approximately 60 amino acids in length, that resemble calcium-binding sites found in other proteins. By binding divalent cations (typically calcium and/or magne-

Table 9-1 Biochemical and Functional Characteristics of Integrins

SUBUNIT (CD, NAME)	KD	LIGANDS
$\alpha_1\beta_1$ (49a/29, VLA-1)	210/130	Laminin, collagen
$\alpha_2\beta_1$ (49b/29, VLA-2)	160/130	Collagen, laminin, ECHO virus
$\alpha_3\beta_1$ (49c/29, VLA-3)	150/130	Collagen, laminin, others
$\alpha_4\beta_1$ (49d/29, VLA-4)	150/130	VCAM-1, fibronectin CS-1 domain, $\alpha_4\beta_1$, $\alpha_4\beta_7$
$\alpha_5\beta_1$ (49e/29, VLA-5)	160/130	Fibronectin
$\alpha_6\beta_1$ (49f/29, VLA-6)	150/130	Laminin
$\alpha_7\beta_1$ (α_7/29)	97/130	Laminin
$\alpha_8\beta_1$ (α_8/29)	180/130	Fibronectin
$\alpha_9\beta_1$ (α_9/29)	130/130	Fibronectin, tenascin
$\alpha_v\beta_1$ (51/29)	135/130	Fibronectin, vitronectin
$\alpha_L\beta_2$ (11a/18, LFA-1)	180/95	ICAM-1, ICAM-2, ICAM-3
$\alpha_M\beta_2$ (11b/18, Mac-1)	170/95	ICAM-1, ICAM-2, C3bi, fibrinogen, heparin
$\alpha_X\beta_2$ (11c/18, p150,95)	150/95	C3bi, fibrinogen
$\alpha_d\beta_2$ (α_d/18)	150/95	ICAM-3
$\alpha_{IIb}\beta_3$ (41/61, gpIIb/IIIa)	120/105	Fibrinogen, other RGD peptides
$\alpha_v\beta_3$ (51/61)	163/105	Vitronectin, PECAM-1, other RGD peptides
$\alpha_6\beta_4$ (49f/104)	150/205	Laminin
$\alpha_v\beta_5$ (51/β_5)	163/100	Vitronectin
$\alpha_v\beta_6$ (51/β_6)	163/106	Fibronectin, tenascin
$\alpha_4\beta_7$ (49d/β_7, ACT-1)	150/105	MAdCAM-1, VCAM-1, fibronectin CS-1 domain
$\alpha_E\beta_7$ (103/β_7, HML-1)	175/105	E-cadherin
$\alpha_1\beta_8$ (49a/β_8)	210/95	Laminin, collagen, fibronectin
$\alpha_v\beta_8$ (51/β_8)	150/95	Laminin, collagen, fibronectin

VLA, Very late antigen; *ECHO*, enterocytopathogenic human orphan virus; *VCAM*, vascular cell adhesion molecule; *CS*, connecting segment; *LFA*, leukocyte function–associated antigen; *ICAM*, intercellular adhesion molecule; *RGD*, arginine-glycine-aspartic acid; *PECAM*, platelet-endothelial cell adhesion molecule.

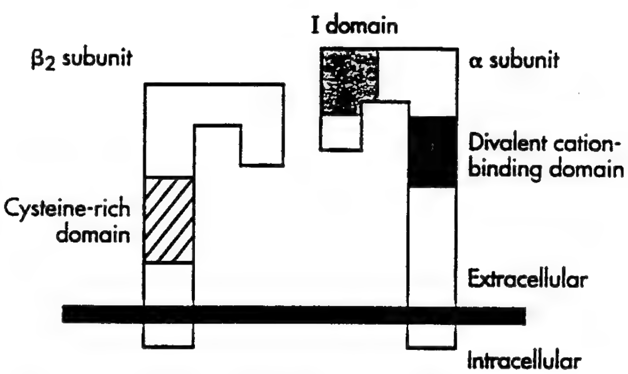

FIGURE 9-3 Schematic representation of a β_2 integrin heterodimer. The I domain and divalent cation-binding domains on the α subunit that contribute to adhesive function are shown, as is the cysteine-rich repeat region of the β_2 subunit that is conserved among integrin β subunits.

sium), these domains contribute to the binding affinity of the heterodimer. Another conserved structural characteristic of many integrins (e.g., all four of the α chains that can associate with β_2 integrin chains, as well as the α_1 and α_2 chains of β_1 integrins) is the presence of an inserted, or "I," domain.[91] This site appears to be an important recognition site for integrin-binding activity. An unusual feature of the extracellular portions of integrin β subunits is the presence of 56 cysteine residues localized into four tandem domains that are felt to keep the heterodimer in an extended, rigid conformation. Expression of integrins is under transcriptional regulation, and analysis of the promoter sequences of several leukocyte integrin genes have identified specific transcription factors that influence expression.[92-94] Intracytoplasmic assembly and subsequent surface expression of integrins requires an intact β subunit because genetic mutations in the β_2 subunit (especially near the N-terminal portion) have been identified in patients with a disorder called *leukocyte adhesion deficiency disease type I* (LAD type I), in which leukocyte surface expression of β_2 integrins is markedly impaired or totally absent.[95,96]

Integrin ligands include other cell-surface adhesion molecules, especially those of the immunoglobulin gene family (see below), complement protein fragments, extracellular matrix proteins, and other molecules (see Table 9-1). Expression of integrins varies tremendously from one cell type to the next. For example, umbilical vein endothelial cells express several β_1 integrins ($\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_5\beta_1$), as well as $\alpha_v\beta_3$;[97] respiratory epithelial cells express the same set of β_1 integrins but also $\alpha_9\beta_1$, $\alpha_v\beta_1$, $\alpha_6\beta_4$, $\alpha_v\alpha_5$, and $\alpha_v\beta_6$.[98] For endothelial and epithelial cells, these receptors are believed to function primarily by mediating adhesion to basement membrane matrix proteins. The pattern of integrin expression on leukocytes and mast cells is also varied (Table 9-2). For example, on eosinophils, $\alpha_4\beta_1$ and $\alpha_6\beta_1$ are expressed,[99] basophils express $\alpha_5\beta_1$ and $\alpha_5\beta_1$, and mast cells express $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$.[100] Other integrin subfamilies are restricted to certain cell types. An example of this is the β_2 integrins, whose expression is restricted to leukocytes.[101] Ligands for β_2 integrins include intercellular adhesion molecule (ICAM-1), ICAM-2, and ICAM-3, as well as fibrinogen, the complement fragment C3bi, and other structures (see Table 9-1). For all leukocytes, the processes of firm adhesion, locomotion, and transendothelial migration are either partially or completely dependent on β_2 integrins. Defects in β_2 integrin expression lead to impaired leukocyte recruitment responses, especially in neutrophils.[95] Among different cell types, however, levels of surface expression vary, and the levels of cell surface expression can be altered during hematopoiesis or as a consequence of cellular activation. For example, the expression of certain β_1 integrins on lymphocytes requires prolonged cellular activation in vitro with mitogens, hence the name very late antigens (VLAs).[102] Other integrins, such as $\alpha_M\beta_2$ and $\alpha_d\beta_2$, exist both on the cell surface and in an intracytoplasmic pool of granules and can rapidly translocate to the cell surface after cell activation.[103] Another aspect of leukocyte integrin expression relates to differences among cell types in stimuli capable of mobilizing these preformed integrins. For example, chemotactic factors such as formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF), and C_{5a} can induce upregulation of $\alpha_M\beta_2$ on eosinophils, basophils, and neutrophils, whereas IL-5 and IL-3 selectively increase $\alpha_M\beta_2$ expression on eosinophils and basophils, respectively.[15,104-107]

As type 1 transmembrane structures, integrins possess intracytoplasmic domains with distinct sites for phosphorylation and for attachment to cytoskeletal elements such as talin, vinculin, α-actinin, filamin, paxillin, and actin.[91,108,109] During adhesion, integrins and associated cytoskeletal proteins localize on the cell surface within contact sites called *focal adhesions*.[110] Specific phosphorylation of the β integrin cytoplasmic domain occurs during cell adhesion or integrin clustering via focal adhesion kinase and other protein kinases.[110-112] Another structural characteristic contributes to the strength of adhesion. There are conserved sequences in the cytoplasmic carboxyl terminus of several β subunits, separate from the phosphorylation sites, that influence the avidity of binding.[91] For $\alpha_4\beta_1$, specific cysteine residues have been identified that are critical for function and structural integrity of the α subunit.[113]

Integrins also have important functions as signal-transducing molecules, mediating so-called "outside-in" signalling.[114,115] For example, integrin-mediated adhesion and activation of focal adhesion kinase can prevent apoptosis.[116] Signalling via integrin clustering does not appear to occur exclusively through the integrins themselves, however, because intracytoplasmic domains of integrins lack kinase or phosphatase activity of their own; they also lack sequence homology with known signalling proteins.[117] Instead, outside-in signalling may occur as a result of interactions between integrins and other associated molecules, such as cytoskeletal proteins.[114,115] Furthermore, integrins such as $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$ associate with other nonintegrin cell surface molecules, such as CD9[118] and members of the transmembrane 4 family of proteins that includes CD53, CD63, CD81, and CD82;[119,120] these co-localized structures may be involved in the regulation of integrin function.

In addition to the level of adhesion molecule expression, it is now

Table 9-2 Surface Expression of Integrins on Human Leukocytes and Mast Cells

SUBUNIT (CD, NAME)	LYMPHOCYTES*	MONOCYTES	NEUTROPHILS	EOSINOPHILS	BASOPHILS	MAST CELLS*
$\alpha_1\beta_1$ (49a/29, VLA-1)	+	−	−	−	−	−
$\alpha_2\beta_1$ (49b/29, VLA-2)	+	+	−	−	−	−
$\alpha_3\beta_1$ (49c/29, VLA-3)	+	−	−	−	−	+
$\alpha_4\beta_1$ (49d/29, VLA-4)	+	+	−	+	+	+
$\alpha_5\beta_1$ (49e/29, VLA-5)	+	+	+	−	+	+
$\alpha_6\beta_1$ (49f/29, VLA-6)	+	+	+	+	−	−
$\alpha_L\beta_2$ (11a/18, LFA-1)	+	+	+	+	+	−
$\alpha_M\beta_2$ (11b/18, Mac-1)	−	+	+	+	+	−
$\alpha_X\beta_2$ (11c/18, p150,95)	+	+	+	+	+	+
$\alpha_d\beta_2$ (α_d/18)	+	+	+	+	+	−
$\alpha_v\beta_3$ (51/61)	−	+	−	−	−	+
$\alpha_4\beta_7$ (49d/β_7, ACT-1)	+	+	−	+	+	−
$\alpha_E\beta_7$ (103/β_7, HML-1)	+	−	−	−	−	−

–, Present; −, absent.
*Expression may be restricted to subsets of these cells.

apparent that conformational changes can occur in integrins, resulting in rapid and reversible changes in binding avidity.[121-123] These changes occur as a result of ligand binding, occupancy of divalent cation binding sites, allosteric changes caused by adjacent cell-surface structures, such as integrin-modulating factor-1, or in association with phosphorylation (e.g., via focal adhesion kinase) of clustered intracytoplasmic domains of the integrin subunits.[91,124] Increased levels of activated β_1 integrins have been detected on leukocytes from patients with chronic inflammatory diseases.[125,126] At least two pathways of cross-talk between G-protein–coupled chemokine receptors and integrins have been identified. Activation by chemokines can lead to differential regulation of integrin avidity,[127-129] perhaps as a result of alterations in nucleotides in the guanosine triphosphate (GTP)-binding protein RhoA.[130] Chemokines can also result in redistribution of integrins in a way that facilitiates their calcium-dependent movement to the leading edge of migrating cells.[131,132]

Among the integrins, the $\alpha_4\beta_1$ heterodimer (VLA-4) is of particular interest in allergic inflammation.[133] This is due in large part to its prominent expression on eosinophils and basophils and its lack of expression on neutrophils,[36] although rat neutrophils[134] and even human neutrophils may be able, under certain conditions, to express α_4 integrins.[135] VLA-4 binds to the alternatively spliced connecting segment-1 (CS-1) portion of the IIICS (type III connecting segment) region of fibronectin (containing the consensus amino acid sequence LDV[136]) and to regions containing the consensus amino acid sequence IDS within the first and fourth domains of vascular cell adhesion molecule-1 (VCAM-1), a molecule expressed on activated endothelial cells.[101,137] It may also function in homotypic binding.[138] Several studies suggest that the avidity of VLA-4 for its ligands differs among cell types and can be dramatically altered by cell activation.[91] VLA-4 is the only integrin that shares with selectins the ability to mediate rolling adhesion under conditions of shear stress.[56,139-141] Another β subunit, β_7, can also pair with α_4 ($\alpha_4\beta_7$) and, like VLA-4, is capable of binding to fibronectin, and VCAM-1,[142,143] but unlike VLA-4, $\alpha_4\beta_7$ binds to another cytokine-inducible adhesion molecule, MAdCAM-1, which is important in homing of lymphocyte subsets (and perhaps eosinophils and basophils, which also express $\alpha_4\beta_7$[15,143,144]) to the gut mucosa.[81] Furthermore, β_7 can pair with an additional subunit, α_E, expressed on lymphocytes (but not granulocytes) where it functions as a ligand for E-cadherin, a molecule found along the basolateral portion of intestinal epithelium.[145]

IMMUNOGLOBULIN GENE SUPERFAMILY

The immunoglobulin gene superfamily (IgSF) of adhesion molecules consists of more than a dozen molecules that have a series of globular domains, formed by disulfide bonds, resembling those found in immunoglobulins.[146] Like integrins, these molecules are responsible for adhesion to other cell-surface ligands and have important signalling functions.

The structures of several important IgSF family members involved in endothelial cell–endothelial cell, endothelial cell–leukocyte, and leukocyte-leukocyte adhesion are shown schematically in Figure 9-4; examples of other IgSF family members include CD2, CD3, CD4, CD8, CD58, major histocompatibility complex (MHC) classes I and II, the T cell receptor, and the sialic acid-binding IgSF subfamily, called *I-type lectins* or *sialoadhesins*, that includes CD22 and CD33.[146-148] ICAM-1 (CD54) was originally discovered as a 90-kD molecule responsible for heterotypic cell adhesion, with a 453–amino acid extracellular domain organized into five Ig-like domains, and putative 24– and 28–amino acid transmembrane and intracytoplasmic domains, respectively.[149,150] Ligands for the most N-terminal domain of ICAM-1 include leukocyte function–associated antigen-1 (LFA-1), fibrinogen, and most serotypes of rhinovirus,[151-154] whereas the third domain is recognized by Mac-1.[155] ICAM-1 is constitutively expressed along the luminal, intercellular, and subluminal surfaces of endothelial cells.[156] Various stimuli, including IL-1, TNF, LPS, and IFN-γ are capable of inducing or enhancing its expression, but IFN-γ selectively induces ICAM-1 expression without affecting expression of other adhesion molecules.[157,158] ICAM-1 expression can be induced on eosinophils,[159,160] as well as other cells, including respiratory epithelial cells.[161,162]

ICAM-2 (CD102) was originally detected as an LFA-1–dependent ICAM-1–independent 60-kD endothelial ligand. It has a 202–amino acid extracellular domain and putative transmembrane and intracytoplasmic domains of 26 amino acids each.[163,164] ICAM-2 has only two immunoglobulin-like extracellular domains that possess 34% homology to the first two domains of ICAM-1.[163] The ligand-binding site for LFA-1 is located in the first N-terminal domain in ICAM-1: peptides from this region have been shown to inhibit endothelial cell adhesion.[165] In addition to endothelial cells, ICAM-2 is constitutively expressed on mononuclear cells, basophils, mast cells, and platelets,[166] and expression is unaffected by cytokines.

ICAM-3 (CD50) also functions as an LFA-1 ligand.[167] It has a range of molecular weights from 116 to 140 kD depending on the cell type studied and possesses 48% to 52% homology to ICAM-1 and 31% to 37% homology to ICAM-2.[167] ICAM-3, like ICAM-1, has five immunoglobulin-like extracellular domains. It is 518 amino acids in length, with a 24–amino acid transmembrane domain, and a 37–amino acid intracytoplasmic domain.[167] ICAM-3 is constitutively expressed on all leukocytes and on mast cells; expression on other cell types, including endothelial cells, has not been detected.[166] ICAM-3 can act as a signalling molecule. Cross-linking results in calcium mobilization, tyrosine phosphorylation, enhanced adhesion, and modulation of basophil mediator release.[168-170]

VCAM-1 (CD106) was identified as a cytokine-inducible endothelial cell structure.[171-173] It can be expressed in two alternatively spliced versions, existing primarily in a seven-domain form (648 amino acids in length) rather than the more rare six-domain form. There is extensive homology between the three N-terminal domains and the fourth through sixth domains, probably a result of gene duplication.[174-176] Both the six- and seven-domain forms have transmembrane regions of

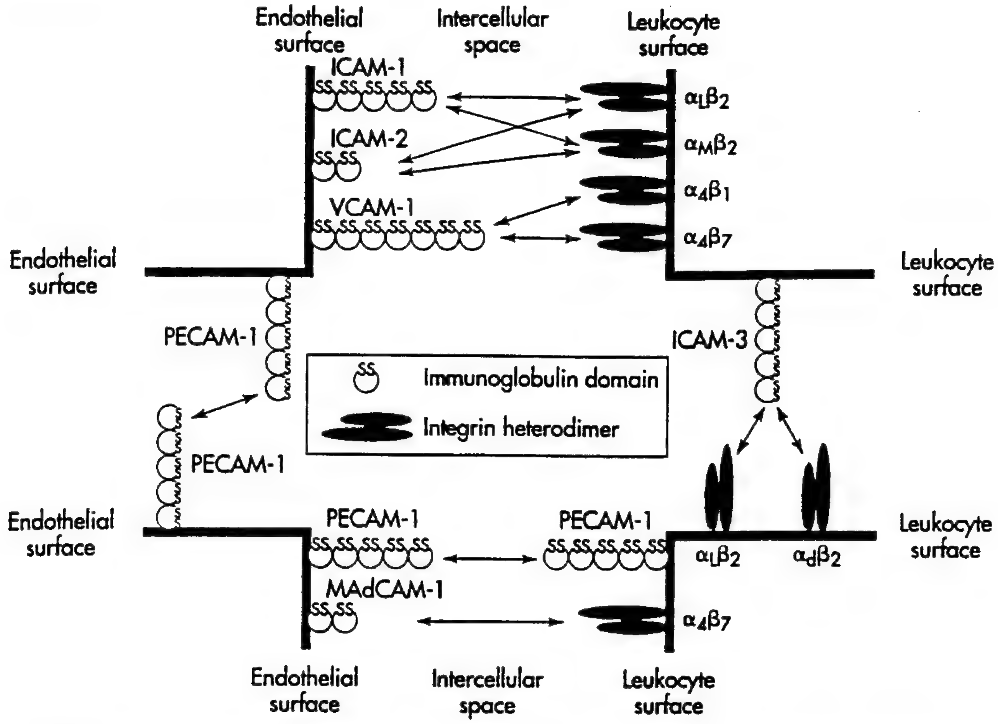

FIGURE 9-4 Schematic representation of several immunoglobulin gene superfamily molecules expressed on endothelial cells and leukocytes. Counterligands, most of which are integrins, are also shown. Arrows denote ligand- counterligand interactions but do not indicate domains used for binding (see text). Note that MAdCAM-1 expression appears to be limited to endothelium in the lamina propria of the gut and to Peyer's patches, whereas others, such as VCAM-1, require specific stimuli to induce expression.

22 amino acids and intracytoplasmic regions of 19 residues. Recently, an even smaller, glycophosphatidylinositol-anchored isoform of VCAM-1 has been detected in murine endothelium.[177,178] Within the extracellular portions of VCAM-1, domains 1 and 4 are most homologous to each other; these are the domains that carry the IDS sequence recognized by VLA-4.[177]

VCAM-1 expression has been detected on cell types other than endothelium, including macrophages, dendritic cells, astrocytes, and bone marrow stromal cells.[179,180] Expression of VCAM-1 on umbilical vein endothelial cells is concentrated primarily on the luminal surface[156] and can be induced de novo within several hours after exposure to IL-1, TNF, or LPS; expression reaches maximal levels by 24 to 48 hours.[172,173,181,182] These treatment conditions lead to increased expression of other endothelial adhesion molecules, including ICAM-1 and E-selectin, via pathways involving proteosomes.[183] In contrast, treatment of endothelial cells with IL-4 or IL-13[184-187] leads to selective induction of VCAM-1 expression, and the combination of IL-4 with TNF is synergistic,[188-190] an effect that is due to transcriptional activation and stabilization of VCAM-1 messenger ribonucleic acid (mRNA).[191] Molecular analysis of the VCAM-1 promoter and cell signalling events suggest that depending on the cytokine stimulus, induction of VCAM-1 expression occurs via NF-κB–dependent and NF-κB–independent pathways, as well as via activation of protein kinase C (PKC) and tyrosine kinases.[192-197] Patterns of VCAM-1 induction may differ among endothelial cell types. For example, human dermal microvascular endothelial cells express VCAM-1 after stimulation with TNF but not IL-1 or IL-4.[198]

Platelet–endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kD molecule with six immunoglobulin domains.[199,200] PECAM-1 is not only constitutively expressed on endothelial cells and platelets but on most leukocytes as well.[166] The transmembrane and intracytoplasmic domains are encoded by multiple exons, and several isoforms can be generated by alternative splicing.[199] PECAM-1 is found at particularly high concentrations at interendothelial cell interfaces, although this may be altered by cytokines.[199,201] Both homotypic and heterotypic adhesion via PECAM-1 have been reported. A specific example of the latter is the interaction of CD31 with the integrin $\alpha_v\beta_3$.[202] Studies with blocking antibodies suggest a critical role for PECAM-1 during transendothelial migration in vitro and in vivo.[200,203]

OTHER ADHESION MOLECULES

Several other adhesion molecules on endothelial cells or leukocytes have been identified; some function during leukocyte recruitment responses. For example, vascular adhesion protein-1 (VAP-1) is a 90-kD sialylated lymphocyte ligand identified in synovial, mucosal, and peripheral lymph node endothelium and at sites of inflammatory disorders but not on unstimulated or activated umbilical vein endothelium.[204-206] A similar molecule is lymphocyte–vascular adhesion protein-2 (L-VAP-2, CD73), a 70-kD structure constitutively expressed on umbilical vein endothelial cells and some lymphocytes; antibody-blocking studies suggest that it also functions as a lymphocyte ligand.[207,208] Another molecule, CD44 (formerly Hermes antigen, H-CAM, or pgp-1), is found at high levels on most leukocytes, endothelial cells, epithelial cells, and other cell types.[209] Many splice variant forms of differing molecular weights have been identified (85 to 160 kD, 90 kD most predominant). This family has been implicated as adhesion molecules for peripheral lymph nodes, hyaluronic acid, and T cell signalling.[209] CD44 has also been shown to mediate interactions between lymphocytes and airway smooth muscle cells, inducing growth of the latter cell type.[210] Eosinophil-priming cytokines such as IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) increase CD44 levels on eosinophils, but the significance of this is uncertain because eosinophils do not bind hyaluronate in vitro.[211] The exact role, if any, of these and other adhesion molecules in allergic inflammation remains to be determined.

ADHESION MOLECULE PHYSIOLOGY: REGULATION OF TETHERING, ROLLING, FIRM ADHESION, AND TRANSENDOTHELIAL MIGRATION BY CYTOKINES, CHEMOKINES, AND OTHER STIMULI

It is generally accepted that a sequence of steps is involved during the emigration of leukocytes from the intravascular compartment into tissue sites.[212] Under the shear forces of blood flow, cells undergo a reversible process during which they "roll" or reversibly attach to the endothelium. These interactions are mediated primarily by carbohydrates and their selectin counterligands. In vitro, adsorbed P-selectin or E-selectin support neutrophil rolling, although E-selectin–mediated rolling rates of leukocytes are slower and more resistant to shear forces.[16,213] It has been suggested that rolling may actually be two separable events, tethering and rolling, where L-selectin on the leukocyte presents oligosaccharide ligands to E-selectin to initiate tethering before stable rolling can occur via other ligand-ligand interactions. Leukocyte rolling can be visualized microscopically in vitro using flow chambers or in vivo in tissues such as rat mesentery.[214] In addition to selectins, the integrin VLA-4 can also participate in cell rolling.[71,169-171]

The next step, firm adhesion, requires leukocyte activation, perhaps as a result of their exposure to leukocyte-activating factors produced by and/or displayed on the surface of endothelial cells, such as PAF[215] or chemokines.[128,216] Associated with these events are increases in both avidity and expression of integrins on the leukocyte surface leading to enhanced binding to ICAM-1 and VCAM-1.[5] Subsequent transendothelial migration, during which time the leukocytes emigrate between endothelial cells and penetrate the basement membrane to enter the extravascular space, is mediated by PECAM-1,[235,242-244] although integrins, selectins, and selectin ligands may also participate.[190,217,218] Additionally, cytokines, chemokines, and other chemotactic factors, by directly activating leukocyte migration responses, can potentiate the processes of adhesion and transendothelial migration.[5,190] Therefore this paradigm would predict that a specific leukocyte infiltrate results from a series of relatively selective recruitment events in which overlapping cell adhesion mechanisms and chemotactic factors function in concert. Consistent with this stepwise recruitment model are studies of patients with genetic defects in human leukocyte β_2 integrins (LAD type I), fucosylation abnormalities resulting in defective generation of selectin ligands (LAD type II),[96] and the phenotype of a variety of single and dual adhesion molecule knockout mice, some of which have subtle changes (e.g., E- or P-selectin knockouts), whereas in others (especially the dual knockouts) impairment of inflammatory responses is profound (Table 9-3).[219-242] Knockouts for other adhesion molecules are lethal and include those for VCAM-1,[243,244] integrins α_4,[245] α_5,[246] and β_1,[247,248] and fibronectin.[249] Finally, there may be tissue-specific exceptions to this paradigm. For example, neutrophil recruitment to the lung in response to bacterial challenge is normal in P-selectin/ICAM-1 dual knockout mice,[96] in mice treated with CD18 antibodies,[250] and in patients with LAD type I,[251] suggesting the presence of recruitment mechanisms independent of CD18, ICAM-1, and P-selectin.

EOSINOPHIL, BASOPHIL, AND MAST CELL INTERACTIONS VIA SELECTINS, INTEGRINS, AND THEIR COUNTERLIGANDS

Functional aspects of human eosinophil, basophil, and mast cell adhesion responses have been the subject of a recent text.[3] Although not necessarily unique to a particular cell type, a number of distinguishing features among cells have been observed. For example, with respect to selectins, both eosinophils and neutrophils bind to cytokine-activated endothelium under rotational conditions in an L-selectin–dependent manner, but neutrophils adhere much greater than eosinophils, in part because they express more L-selectin.[55] Surprisingly, one L-selectin antibody, LAM 1-11, selectively inhibited eosinophil but not neutrophil adhesion under these conditions, suggesting the presence of

Table 9-3 Manifestations of Viable Adhesion Molecule Deficiency States in Humans and Knockout Mice

DEFICIENCY	PHENOTYPE AND CONSEQUENCES
Human	
LAD type I	Blood neutrophilia with tissue neutropenia, delayed umbilical cord separation, recurrent soft tissue infections, impaired pus formation, and wound healing; reduced or absent neutrophil adhesion, transendothelial migration, and chemotactic responses; normal rolling adhesion
LAD type II	Severe mental retardation, short stature, distinctive facial appearance, Bombay (hh) blood phenotype, impaired pus formation, recurrent pneumonia, periodontitis, otitis, and cellulitis; neutrophils have reduced or absent sLe^x expression, reduced rolling adhesion, normal firm adhesion
Mouse (knockouts)	
ICAM-1	Impaired leukocyte recruitment to inflamed peritoneum and to sites of contact sensitivity, neutrophilia, lymphocytosis, improved resistance to LPS-induced shock, protection from ischemic, cerebral, and renal injury, improved cardiac allograft survival
$\alpha_1\beta_1$ (VLA-1, CD49a/CD29)	Normal, but defects in adhesion to collagen can be demonstrated with some cells
β_2 integrin (CD18)	Impaired leukocyte recruitment to inflamed peritoneum and to sites of contact sensitivity, neutrophilia, lymphocytosis; some strains developed psoriasis-like skin disease
LFA-1 (CD11a/CD18)	Impaired tumor rejection but preserved cytotoxic T lymphocyte responses
β_7 integrin	Impaired formation of gut-associated lymphoid tissues
CD34	Normal except reduced allergen-induced eosinophil accumulation
L-selectin	Markedly reduced leukocyte rolling and recruitment to inflamed peritoneum and to sites of contact sensitivity, improved resistance to LPS-induced shock, small lymph nodes, splenomegaly, normal antibody production; impaired primary T cell responses
E-selectin	Normal; profound impairment of neutrophil recruitment after infusion of P-selectin mAb
P-selectin	Absent leukocyte rolling, neutrophilia, delayed neutrophil recruitment to inflamed peritoneum and to sites of contact sensitivity
Dual ICAM-1/P-selectin	Complete blockade of neutrophil recruitment during bacterial-induced peritonitis; no inhibition of neutrophil recruitment during bacterial-induced pneumonitis; blood leukocyte counts similar to ICAM-1 knockouts; no leukocyte rolling after traumatic injury
Dual E-selectin/P-selectin	Complete blockade of neutrophil recruitment during bacterial-induced peritonitis; no inhibition of neutrophil recruitment during bacterial-induced pneumonitis; blood leukocyte counts similar to ICAM-1 knockouts; severely impaired leukocyte rolling and recruitment in response to infection or cytokine-induced meningitis; altered hematopoiesis

unique functional epitopes on eosinophil L-selectin.[55] Basophils, like other granulocytes, shed L-selectin on activation, but the shedding is less complete;[107,252] its function on basophils has not been studied.

Eosinophils express a form of the P-selectin ligand, PSGL-1, that is 10 kD greater in size than on neutrophils. Other differences include higher levels on eosinophils and the presence of the 15-decapeptide repeat form instead of the 16-decapeptide repeat form found on neutrophils.[69] Eosinophils attach as well as or better than neutrophils to P-selectin immobilized on plastic surfaces,[46,253] in tissue sections from nasal polyps,[254] or in in vitro rolling assays.[69] Whether basophils express PSGL-1 and have similar interactions with P-selectin is not known, although mouse mast cells derived from bone marrow cultures will roll on P-selectin.[255]

Eosinophils and basophils, like neutrophils, bind to E-selectin.[14,15] Their adhesion depends on leukocyte surface expression of sialic acid because removal of sialic acid by treatment with neuraminidase abolishes all adhesive activity.[14,15] Basophils bind best to E-selectin and eosinophils bind at the lowest levels, findings that correlate with the quantity of sialyl-dimeric Le^x, not sLe^x, on the cell surface.[14,15] Further evidence for the role of extended-chain carbohydrates as E-selectin ligands comes from results of experiments utilizing endo-β-galactosidase, an enzyme that removes extended forms of sLe^x such as sialyl-dimeric Le^x. Enzyme treatment almost completely inhibits binding of all three cell types, suggesting that sialyl-dimeric Le^x, not merely sLe^x, is responsible for E-selectin adhesion.[14,15] Preliminary studies have identified sialylated ligands for E-selectin on glycolipids extracted from normal human eosinophils and neutrophils.[76]

Another important selective adhesion pathway involves interactions between α_4 integrins and VCAM-1. Ever since the mid-1980s, it has been known that cytokine-activated monolayers of cultured umbilical vein endothelial cells acquire enhanced adhesiveness for neutrophils, eosinophils, and basophils.[26-28,36,256,257] Antibodies to CD18, ICAM-1, and E-selectin were shown to inhibit adherence of all three leukocyte types.[34,36,256,257] However, important differences were noted in adhesion kinetics among these cell types: longer cytokine incubations (24 to 48 hours) led to loss of neutrophil adhesion but maintenance of eosinophil and basophil adhesion.[29,256,257] Around the same time, histopathologic studies of biopsy sites from patients with LAD type I revealed an absence of tissue neutrophilia but obvious tissue eosinophilia.[251] It soon became clear, with the discovery of VCAM-1 and its prolonged expression induced by cytokines,[172] that this molecule might explain the differences in adhesion among these cells. Anti–VCAM-1 antibody (or antibody to VLA-4, its counterligand) was effective in inhibiting eosinophil adherence but had no effect on neutrophil adherence.[35,36,258,259] In these studies, basophil adherence was also demonstrated to be partly mediated through VCAM-1, although the inhibitory effect seen with anti–VCAM-1 antibody was less impressive.[36] The ability of eosinophils and basophils to adhere to VCAM-1 via VLA-4 was directly confirmed by showing that these cells could adhere to an immobilized recombinant form of VCAM-1 and in experiments in which the adhesion was inhibited using VCAM-1 or VLA-4 antibodies.[15,35,185] Subsequent studies revealed that selective induction of VCAM-1 expression on endothelial cells by IL-4 or IL-13 did not influence neutrophil adherence but did induce eosinophil and basophil adherence in a VCAM-1/VLA-4–dependent manner.[185,187] These findings are consistent with several IL-4–related studies in animals: (1) intraperitoneal or intradermal injection of mice with IL-4 caused an eosinophil-rich infiltrate,[260] (2) IL-4 transgenic mice developed tissue eosinophilia and an allergic-like syndrome,[261] (3) mice inoculated with an IL-4 transfected tumor cell line developed local eosinophilia at the tumor site,[262] (4) transgenic mice expressing IL-4 locally in the lung developed pulmonary eosinophilia,[263] and (5) anti–IL-4 reduced antigen-induced expression of VCAM-1 in mouse trachea and eosinophil recruitment to the lung.[264,265] Therefore selective induction of VCAM-1 expression by certain cytokines may contribute to the preferential recruitment of eosinophils (and possibly basophils) seen during certain inflammatory responses. In addition, the discovery that eosinophils and basophils, but not neutrophils, express $\alpha_4\beta_7$,[15,144] a molecule that recognizes both VCAM-1 and MAdCAM-1,[15,81,143] suggests that this integrin may also play a role in preferential recruitment responses. However, α_4 integrins are expressed on other cell types, including lymphocytes, monocytes, and mast cells,[266,267] and there are situations in vivo in which acute or chronic eosinophil accumulation occurs without detectable endothelial VCAM-1 expression[34,268,269] or under conditions in which VCAM-1 is expressed at relatively high levels but little or no eosinophil accumulation is seen,[270-272] so it appears that the VCAM-1/VLA-4 adhesion pathway cannot solely explain selective eosinophil and basophil recruitment.

Several in vitro studies have begun to analyze the molecular mechanisms regulating eosinophil transendothelial migration and have identified characteristics that distinguish eosinophils from neutrophils.[190] Treatment of endothelial monolayers with IL-1 or TNF increased eosinophil transendothelial migration in a CD18-dependent manner.[273,274] However, a combination of VCAM-1, ICAM-1, and E-selectin antibodies was more effective than ICAM-1 antibody alone.[274] Eosinophil-priming cytokines such as GM-CSF or IL-5 markedly potentiate their transendothelial migration across unstimulated or cytokine-activated endothelial cell monolayers.[190] Activation of β_1 integrins on eosinophils by incubation with an antibody that activates its function stimulated adhesion but inhibited eosinophil transendothelial migration, presumably by preventing de-adhesion needed for migration.[275] Thus far the role of PECAM-1 in eosinophil transmigration remains unknown, and mechanisms of basophil transendothelial migration have not been examined.

Several stimuli including cytokines possess the ability to selectively enhance eosinophil or basophil adhesion-related responses. For example, exposure of eosinophils to IL-3, IL-5, or GM-CSF augments adhesion molecule function, induces L-selectin shedding and CD11b up-regulation, and enhances chemoattractant-induced adhesion responses and transendothelial migration, with little or no effect on neutrophils.[105,106,276-282] For basophils, IgE-dependent degranulation or treatment with IL-3 will enhance adhesion to endothelial cells in a β_2 integrin-dependent manner.[104,283] Exposure to the C-C chemokine regulated in activation, normal T cell expressed and secreted (RANTES), a potent and selective eosinophil activator and chemoattractant in vitro[284] and in vivo,[285,286] causes eosinophil transendothelial migration.[287] The effects of RANTES on eosinophils are synergistic with IL-5 in promoting CD18- and VLA-4–dependent transmigration across IL-1–activated endothelium; a similar potentiated RANTES response is seen with eosinophils from bronchoalveolar lavage.[287] It also appears that C-C chemokines are potent activators of basophil migration and degranulation.[284] In each instance, these stimuli have little or no effect on neutrophils. Furthermore, eosinophil-activating cytokines and chemokines have been detected at sites of allergic inflammation,[288-290] and both epithelial cells and endothelial cells have been shown to produce RANTES and other eosinophil-active chemokines.[291-294]

Cytokines and chemokines may also be important in regulating the functional state of integrins. For example, β_1 integrins on eosinophils exist in a state of partial activation and can be maximally activated for adhesion to VCAM-1 or fibronectin after exposure to certain divalent cations (e.g., Mn^{2+}) or integrin-activating antibodies, conditions that do not affect the total cell surface expression of β_1 integrins.[295,296] In contrast, IL-5 prevents cation-induced β_1 integrin activation,[295] as did the tyrosine kinase inhibitor tyrphostin.[296] Enhanced adhesion of eosinophils from asthmatic patients to VCAM-1 and ICAM-1 in vitro has been reported.[297] These data suggest that cytokines and chemokines can activate certain adhesion pathways while inactivating others. This, in fact, has been reported to occur in hematopoietic cell lines, monocytes, and eosinophils.[129,298,299] Taken together, these data suggest that C-C chemokines with eosinophil and basophil chemotactic activity, especially in the presence of priming cytokines, may significantly contribute to their selective transmigration in vivo.

Once leukocytes enter the extravascular space, migration through the basement membrane and tissue parenchyma is influenced by interactions with extracellular matrix proteins.[91] This may also hold true for mast cells and their localization within tissues.[267] Most of the receptors for matrix proteins on eosinophils, basophils, and mast cells belong to the β_1 integrin family (see Tables 9-1 and 9-2). Eosinophils can bind to fibronectin via α_4 integrins,[300,301] although activation is required for optimal binding.[296] Eosinophils can also bind to laminin via α_6 integrins.[99] Adhesion to fibronectin (or VCAM-1) via VLA-4 activates a variety of eosinophil functions,[302,303] including production of superoxide anion,[304,305] leukotriene release,[301] and production of GM-CSF and perhaps other cytokines that augment eosinophil survival in an autocrine fashion.[306,307] Degranulation of adherent eosinophils

may be augmented[300] or inhibited,[308] depending on the substrate. Finally, many eosinophils trafficking through the lung ultimately undergo transepithelial migration before appearing in the airway lumen. Cytokines such as TNF, and viral infection of epithelial cells, increase eosinophil–epithelial cell adhesion; the adhesion pathways involved are not entirely clear because this is only partially inhibited by antibodies to ICAM-1 or β_2 integrins.[98,309-311]

Among β_1 integrins, human basophils express α_4 and α_5 integrins that mediate adhesion to VCAM-1 and/or fibronectin.[312] Human skin mast cells, like basophils, express α_4 and α_5 integrins but also α_3 integrins. The latter is used for adhesion to and migration on laminin, whereas all three are capable of mediating fibronectin binding.[100,267] It has been suggested that mast cell interactions with laminin may be important in tissue localization in vivo.[313] Basophils from asthmatic (but not normal) donors release histamine on antibody cross-linking of β_1 integrins, yet IgE-dependent basophil and mast cell mediator release is inhibited by such cross-linking.[314] Using rat, mouse, or culture-derived human mast cells, interactions with fibronectin enhance IgE-dependent histamine and cytokine release.[315,316] Thus integrin engagement can affect a wide range of biologic activities on eosinophils, basophils, and mast cells.

EXPRESSION AND FUNCTION OF ADHESION MOLECULES IN VIVO DURING ALLERGIC INFLAMMATORY RESPONSES

The potential role of adhesion molecules in allergic disease pathophysiology has been studied using a number of different approaches.[37,317,318] One approach focuses on the detection of endothelial activation at sites of allergic inflammation. For example, the expression of endothelial adhesion molecules has been examined immunohistochemically in the skin, nose, and lower airways after experimental allergen challenge, as well as in allergic and other eosinophilic diseases. With respect to allergen challenge studies, intradermal injection of allergic subjects with allergen activates endothelial expression of E-selectin and VCAM-1 and increases expression of ICAM-1.[34,319-321] E-selectin expression induced in this situation was inhibited when a biopsy of the site was immediately performed and it was placed into culture with a mixture of antibodies that neutralize IL-1 and TNF.320 Increases in VCAM-1 was also observed 24 hours after local intranasal allergen challenge, with numbers of infiltrating eosinophils modestly correlating with the extent and intensity of VCAM-1 staining.[322] Endobronchial allergen challenge resulted in increased endothelial VCAM-1 staining and epithelial ICAM-1 staining with a significant correlation between these parameters and eosinophil influx,[323] whereas ocular challenge increased ICAM-1 expression on conjunctival epithelium.[324] In nonhuman primates, allergen inhalation resulted in E-selectin expression on the airway vascular endothelium within 6 hours.[325] There is additional indirect evidence that endothelial activation also occurs within the human airway after intrabronchial allergen challenge because increased levels of soluble forms of E-selectin, ICAM-1, and VCAM-1 are observed in BAL fluids[252,326,327]; in one of these studies there was a correlation with eosinophil influx and levels of both IL-4 and IL-5.[327]

In addition to endothelial changes, altered expression of adhesion molecules occurs on eosinophils and basophils during their allergen-induced movement from the circulation into tissues. For example, levels of adhesion molecules on granulocytes recovered from blood and either sputum, bronchoalveolar lavage, or nasal lavage after antigen challenge revealed increased expression of CD11b[252,328-331]; diminished levels of L-selectin,[252,331,332]; and little or no change in expression of LFA-1, CD32, or VLA-4.[252,328,330]

Along with experimental allergen challenge studies, cell adhesion molecules have been implicated in the pathophysiology of allergic rhinitis and asthma. In perennial rhinitis, studies of nasal airway tissue detected increased expression of ICAM-1 and VCAM-1, but not E-selectin, compared with tissues from nonallergic controls.[333] Seasonal exposure to pollen led to increases in nasal epithelial cell expression of ICAM-1 along with increased numbers of eosinophils, neutrophils, and metachromatic cells.[334] In asthma, studies are somewhat contradictory perhaps because of differences in patient severity and/or treatments. One study examining bronchial mucosal biopsies from normal subjects and mild allergic asthmatic subjects found similar levels of endothelial ICAM-1 and E-selectin despite an increased number of eosinophils in the mucosa of the asthmatic subjects.[268] Treatment with inhaled corticosteroids reduced the tissue eosinophilia without changing ICAM-1 or E-selectin expression. A subsequent study compared endothelial adhesion molecule expression in airway biopsies from subjects with mild allergic and nonallergic asthma, as well as normal controls.[323] Constitutive expression of ICAM-1, VCAM-1, and E-selectin was observed in all groups. Endothelial staining for ICAM-1 and E-selectin, not VCAM-1, was significantly increased in the nonallergic asthmatic group only, whereas epithelial staining for ICAM-1 was increased in both groups of asthmatic subjects. A third study comparing normal subjects to both allergic and nonallergic asthmatic subjects found increased epithelial ICAM-1 and increased endothelial ICAM-1, E-selectin, and VCAM-1, but only in the allergic asthmatic subjects.[335] Analysis of the nonallergic asthmatic subjects was complicated by the inclusion of subjects with more severe disease and higher medication requirements. Correlations were seen between eosinophil infiltration and endothelial ICAM-1 and E-selectin, whereas the correlation with endothelial VCAM-1 did not quite reach statistical significance. In two other studies of moderately symptomatic asthmatic subjects, strong endothelial staining for VCAM-1, as well as for ICAM-1, was observed and correlated with levels of IL-4 in the airway.[336,337] Increased serum levels of soluble ICAM-1 and E-selectin, but not VCAM-1, have been measured in patients admitted for exacerbations of asthma,[318] and, in another study, increased levels of soluble VCAM-1 were reported in asthmatic subjects.[338] Endothelial activation also occurs in atopic dermatitis, as documented by histologic evidence of endothelial adhesion molecule expression[339,340] and increased serum levels of soluble E-selectin.[341] Further support for the role of VCAM-1 in eosinophilic inflammation was provided by the demonstration of VCAM-1 staining of blood vessels, without E-selectin staining, in skin of patients with eosinophilic vasculitis,[342] and significant VCAM-1 staining in nasal polyps, tissues in which extensive eosinophilia is seen.[293,343,344] At least two studies have implicated TNF as a possible inducer of VCAM-1 in the nasal mucosa.[344,345]

Direct proof of adhesion molecule involvement in allergic diseases will, by necessity, require the use of specific adhesion molecule antagonists.[346] Although no data exist for allergic diseases in humans, antibodies to adhesion molecules have been used in some clinical trials.[101] These efforts have been motivated in large part by success seen in animal studies. Blocking monoclonal antibodies have been infused in vivo in a variety of animal models of allergic inflammatory conditions of the airways and skin[264,325,347-357] (Table 9-4). Some-

Table 9-4 Examples of Adhesion Molecule Antibody Treatments Tested in Animal Models of Allergic Diseases

INFLAMMATORY MODEL, SPECIES	ANTIBODY
Antigen-induced eosinophil recruitment and airway responsiveness	
Monkey	ICAM-1[347, 348]
Rabbit	VLA-4[349]
Antigen-induced eosinophil recruitment and allergic late phase responses	
Sheep	VLA-4[350]
Guinea pig	CD18[351, 352] VLA-4[352, 353]
Antigen-induced T cell and eosinophil recruitment to trachea, mouse	VLA-4, VCAM-1, ICAM-1, CD11a[264]
Antigen-induced airway responses, rat	CD11a, CD11b[354] VLA-4[354, 355]
Antigen-induced neutrophil recruitment and allergic late phase responses, monkey	E-selectin, ICAM-1[325]
Chronic eosinophilic airway inflammation, monkey	ICAM-1[356]
Passive cutaneous anaphylaxis-induced eosinophil influx, guinea pig	VLA-4[357]

times, infusion of adhesion molecule antibodies failed to inhibit cell influx yet still resulted in "clinical" benefit, perhaps because of effects on cell function.[350,358] In addition to antibodies, a wide variety of novel pharmaceutical approaches are being tested for their ability to prevent cell recruitment responses by blocking expression and/or function of adhesion molecules.[359-367] Ultimately, however, information on the role of adhesion molecules in allergic diseases in vivo must await studies with antagonists in humans.

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J.E.M.
Subm. 6)

$\alpha d\beta 2$ integrin is expressed on human eosinophils and functions as an alternative ligand for VCAM-1

Mitchell H. Grayson, Monica Van der Vieren*, Sherry A. Sterbinsky, W. Michael Gallatin*, Patricia A. Hoffman*, Don Staunton*, and Bruce S. Bochner[1].

The Johns Hopkins University School of Medicine, Division of Clinical Immunology, Baltimore, MD, and *ICOS Corporation, Bothell, WA.

[1]Corresponding author:

Bruce S. Bochner, M.D.

Johns Hopkins Asthma and Allergy Center

5501 Hopkins Bayview Circle

Baltimore, MD 21224

phone: 410/550-2131

fax: 410/550-2130

email: bbochner@welchlink.welch.jhu.edu

Running title: $\alpha d\beta 2$ on eosinophils binds VCAM-1

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Summary

The β2 family of integrins, CD11a, CD11b, CD11c, and αd, are expressed on most leukocytes. We show that the newest member of this family, αd, is expressed on human eosinophils in peripheral blood and at higher levels on eosinophils in late-phase allergen challenge BAL fluid. Surface expression on eosinophils can be upregulated within minutes by phorbol ester or calcium ionophore A23187. Culture of eosinophils with IL-5 leads to a 2-4 fold increase in αd levels by 3-7 days without a change in α4 integrin expression. Regarding αdβ2 ligands, in both freshly isolated and IL-5 cultured eosinophils, as well as αdβ2 transfected CHO cells, αdβ2 can function as a ligand for VCAM-1. This conclusion is based in part on the ability of mAbs to αd, β2, or VCAM-1 to block cell attachment in static adhesion assays. More specifically, adhesion to VCAM-1 appears to be primarily α4 integrin-dependent in fresh eosinophils, with a smaller αd integrin-dependent component, while adhesion of IL-5 cultured eosinophils to VCAM-1 is equally dependent on α4 and αd integrins. Based on the ability of a VCAM-1 blocking mAb to inhibit αdβ2-dependent CHO cell adhesion, this interaction appears to occur in the first domain of VCAM-1. These data suggest that αdβ2 is an alternative ligand for the first domain of VCAM-1, and may play a role in eosinophil adhesion to VCAM-1 in states of chronic inflammation.

Introduction

Eosinophils have been shown to play an important role in a variety of inflammatory diseases (1). Besides their postulated importance in parasitic infections, these cells are felt to participate in the pathogenesis of allergic disease. In asthma, for example, eosinophils are selectively recruited into the lung, where release of their products contributes to the airways damage that is seen in asthma (2, 3). Indeed, one of the possible mechanisms by which corticosteroids work in asthma is that they substantially decrease eosinophil numbers both in the lung and peripheral circulation (4, 5).

Integrins are a class of heterodimeric surface molecules involved in cellular adhesion (6). They are expressed on leukocytes and other cells, and are composed of both an α and β chain. Based on shared β subunits these molecules can be classified into families. Eosinophils express members of the $\beta 1$, $\beta 2$, and $\beta 7$ integrin families, and in many respects, their integrin expression resembles that of other leukocytes (7). However, because human eosinophils express $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins, but neutrophils do not, their interaction with one of their ligands, VCAM-1, is felt to be a mechanism by which selective recruitment of eosinophils into sites of allergic inflammation occurs (8-10).

Within the $\beta 2$ integrin family, eosinophils, like neutrophils, express CD11a, CD11b, and CD11c (11). Recently a fourth $\beta 2$ integrin, $\alpha d\beta 2$, was identified and found to be most homologous to CD11b/CD18 and CD11c/CD18 (12). $\alpha d\beta 2$ is expressed on most human leukocytes including neutrophils, monocytes, and, to a lesser extent, lymphocytes (11, 12). Van der Vieren, et al., using $\alpha d\beta 2$-expressing chinese hamster ovary (CHO) transfectants, demonstrated binding of $\alpha d\beta 2$ to a human ICAM-3 chimeric protein (12). Whether $\alpha d\beta 2$ is expressed on eosinophils, and how it functions on these cells, was not examined.

The goal of the present studies was to examine the expression and function of $\alpha d\beta 2$ integrins on human eosinophils. We report that eosinophils express $\alpha d\beta 2$, that its surface expression can be acutely and chronically regulated by various stimuli, and, like $\alpha 4$ integrins, can function as a ligand for VCAM-1.

Methods

Reagents

The following murine IgG_1 monoclonal antibodies were used: irrelevant control IgG_1 mAb (Coulter Cytometry, Hileah, FL), CD11a mAb (MHM24, courtesy of Dr. James Hildreth, Johns Hopkins University School of Medicine, Baltimore, MD (13)), CD11b mAb (H4C2, Dr. Hildreth (14); and clone 44, R & D Systems, Minneapolis, MN), CD11c mAb (BU-15, Immunotech, Westbrook, ME), αd mAb (169A, non-blocking, used for flow cytometry (12); 240I, used in adhesion assays because of its blocking ability (Staunton D., manuscript in preparation)), CD18 mAb (H52, Dr. Hildreth (15); and 7E4, Immunotech), α4 (CD49d) mAb (HP2/1, Immunotech), CD16 mAb (3G8, Medarex, Inc., West Lebanon, NH), and blocking $F(ab')_2$ anti-VCAM-1 mAb (IG11b1, Caltag Laboratories, Burlingame, CA). Also used was an IgG_{2a} FITC-anti-CD9 mAb (3B5, Coulter), polyclonal human IgG (Sigma Chemical Co., St. Louis, MO), R-phycoerythrin (PE)-conjugated $F(ab')_2$ goat-anti-mouse IgG (BioSource International, Camarillo, CA), murine polyclonal IgG (Sigma), and FITC-conjugated polyclonal goat anti-human IgE (Kierkegaard and Perry, Gaithersburg, MD). Soluble, recombinant human VCAM-1 and E-selectin (R & D Systems), and bovine serum albumin (BSA, Sigma), were also purchased.

The following stimuli were used: phorbol myristate acetate (PMA) and the calcium ionophore A23187 (Sigma). Several C-C chemokines were also used, including macrophage derived chemokine (MDC, Gryphon, San Francisco, CA), RANTES and Eotaxin (R & D Systems).

Cell isolation

Normodense (s.g. $\geq$ 1.090) eosinophils were isolated from peripheral blood of allergic volunteers by density gradient centrifugation, hypotonic erythrocyte lysis, and

immunomagnetic negative selection as previously described, while neutrophils were purified from peripheral blood of normal volunteers using density gradient centrifugation and hypotonic erythrocyte lysis alone (16, 17). Respective purities always exceeded 95%. Enrichment of peripheral blood for basophils was performed using a double-percoll density gradient seperation, increasing the number of basophils to 3-10% of the total leukocyte count (18).

In some experiments purified eosinophils were cultured for up to 7 days in RPMI 1640 (Biofluids, Inc., Rockville, MD) with 1% L-glutamine, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 500 ng/ml amphotericin (Life Technologies, Gaithersburg, MD), supplemented with 10 ng/ml recombinant human IL-5 (R & D Systems) as described (19). Viability after 1 day or less of culture was ≥ 95%, whereas viability by 7 days was 80±1% (mean±sem, n=2). Culture preparations in which ≤ 50% of cells were viable were excluded from analysis (5 of 42 experiments). In other experiments, eosinophils were incubated with optimal concentrations of various stimuli (50 ng/ml PMA or calcium ionophore A23187, 100 nM MDC, 100 ng/ml RANTES, or 100 μM Eotaxin in PBS/0.1%BSA) for up to 15 minutes at 37°C.

Bronchoalveolar lavage (BAL) cells were obtained from allergic patients who had undergone an endobronchial segmental allergen challenge with either ragweed or *D. pterynissinus* extract 18 hours previously as described elsewhere (20). Eosinophil purity in the late phase BAL fluid was 19±4% (mean±sem, n=5).

CHO transfectants

Chinese hamster ovary cells were transfected with both the human αd and β2 integrin chains as previously described (12). αdβ2-transfected CHO cells were cultured in DMEM/F12 media with 1 mM pyruvate and 2 mM L-glutamine (Biofluids) supplemented

with 10% dialyzed FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 600 μg/ml G418 (all from Life Technologies). Media for culture of the parental CHO cell line was similar except that non-dialyzed FBS (Life Technologies) was used and 0.1 mM hypoxanthine and 16 nM thymidine (Sigma) were used in place of the G418.

Flow cytometry

Expression of integrins on the CHO cell transfectants or on freshly isolated cells from blood following stimulation or culture, was evaluated using single color indirect immunofluorescence and flow cytometry as previously described (18, 21). Dual color detection of basophils (using anti-IgE) and lower purity eosinophils in BAL fluids (using anti-CD9) was also performed. All samples were fixed in 0.1% paraformaldehyde (Sigma) and analyzed using an EPICS Profile II flow cytometer (Coulter). Approximately 10,000 events were collected and displayed on a 4-log scale yielding values for mean fluorescence intensity (MFI).

Adhesion assays

For eosinophils, both freshly purified and cultured, ^{51}Cr-labelled cell adhesion to VCAM-1 (250 ng/ml) or BSA (1%) coated wells was performed for 30 min, at 37°C as previously described (22). In some experiments, cells were preincubated for 30 min, at 4°C with saturating concentrations of one or more of the following blocking mAbs prior to examining their adhesion: CD18 (7E4), CD11a (MHM24), CD11b (clone 44), CD11c (BU-15), αd (240I), and α4 integrin (HP2/1).

For transfected and parental CHO cells, adhesion was performed using coated plates identical to those employed for eosinophil adhesion. However, because the interaction between CHO transfectants and VCAM-1 was not as strong as that between eosinophils and VCAM-1 (data not shown), a modification of a previously described (23)gentle

washing technique was employed. This technique allowed non-adherent cells to be dislodged from the inverted plate at 1g for 30 min at 20°C. Remaining adherent cells were then removed using 0.1 M EDTA (Sigma) and counted by flow cytometry. The percent adhesion was determined from the number of adherent cells as compared to the total number of cells added. In addition to VCAM-1, E-selectin (100 ng/ml) was also used to coat wells in some adhesion experiments. Besides the blocking mAbs used in the eosinophil studies, in certain experiments plates were pretreated with an appropriate dilution of $F(ab')_2$ anti-VCAM-1 mAb prior to the addition of CHO cells.

Statistical analyses

Statistical analyses were performed using an analysis of variance (ANOVA) with a Fisher post-hoc t-test. Significance was set at $p < 0.05$ for all tests.

Results

Expression of αd integrin on human granulocytes

As shown in Figure 1a, eosinophils express all four of the $\beta 2$ integrins, including $\alpha d\beta 2$. The level of surface expression of αd integrin was greater than that of CD11c, but less than expression of $\alpha 4$ integrin (CD49d), CD11a, or CD11b.

Most peripheral leukocytes express αd integrin, with monocytes and a subpopulation of CD8+ lymphocytes having the highest levels (11, 12). As shown in Figure 1b, eosinophils and neutrophils have roughly similar levels of expression, while basophils have slightly higher levels of αd integrin expression.

Regulation of αd integrin surface expression on human eosinophils

Initial studies were performed to determine whether eosinophils could rapidly mobilize intracellular stores of $\alpha d\beta 2$ as has been reported for neutrophils (12). Purified peripheral blood eosinophils were incubated for 15 min, with either PMA or calcium ionophore, A23187, and the surface expression of several α chains of the $\beta 2$ integrins was then measured by indirect immunofluorescence. Figure 2 shows the kinetics of this upregulation with phorbol ester. Both PMA (50 ng/ml) and calcium ionophore (1 μM. data not shown) significantly increased the expression of αd integrin and CD11b. Within minutes of adding PMA, expression increases, reaching significantly increased levels by 10 min. Therefore, eosinophils appear to have preformed stores of $\alpha d\beta 2$ which, similar to CD11b stores, can be rapidly mobilized to the cell surface. Other eosinophil-active stimuli were tested for their acute effects on $\alpha d\beta 2$ expression. Incubation of eosinophils for 15 min with MDC (100 nM), IL-5 (10 ng/ml), RANTES (100 ng/ml), and Eotaxin (100 μM) failed to alter αd integrin expression (data not shown).

Many eosinophil responses can be enhanced by prolonged exposure to certain cytokines, such as IL-5, a phenomenon referred to as "priming" (24). We therefore determined whether eosinophil culture with IL-5 would lead to changes in surface expression of αd integrin. Figure 3a is a representative histogram showing surface expression of αd and α4 integrins from the same eosinophil preparation both before and after 4 days of culture with 10 ng/ml IL-5. While the level of αd integrin increased 4-5 fold, the level of α4 integrin remained unchanged. The kinetics of this increase in αd integrin expression is shown in Figure 3b. As can be seen, the level of αd integrin increases, with statistically significant increased levels at days 4-7 of culture. In contrast, levels of α4 integrin did not change significantly. Because late phase BAL eosinophils express many characteristics of cytokine-primed eosinophils (25, 26), their levels were also compared. Indeed, late phase BAL eosinophils also showed a statistically significant increased level of αd integrin expression, with levels similar to those seen after 3 days of culture in IL-5 (Figure 3b, right side).

αd integrin binds to VCAM-1

Although αd integrin has been shown to bind ICAM-3 and mediate leukocyte-leukocyte adhesion (12), the next series of experiments were designed to examine other possible αd ligands for eosinophils. In part because of previous studies suggesting β2 integrin dependent, CD11b independent eosinophil adhesion to VCAM-1 ((22) and unpublished observations), initial studies were performed using immobilized recombinant VCAM-1.

As shown in Figure 4a, freshly isolated eosinophils adhered to VCAM-1, and mAb blockade of α4 integrin effectively inhibited adhesion, while CD11b blockade had no effect. However, adhesion could also be significantly and consistently inhibited by the αd mAb 240I, albeit to a lesser degree (≈30% inhibition). Even more striking were results of VCAM-1 adhesion experiments in which IL-5 cultured eosinophils, expressing enhanced

levels of αd integrin, were employed. Data in Figure 4b shows that under these conditions, mAbs to CD18, αd, or α4 integrins were equally effective in reducing adhesion to background levels, while a combination of blocking mAbs to CD11a, CD11b, and CD11c had no effect. Note also that IL-5 cultured eosinophils displayed enhanced background adhesion and reduced VCAM-1 adhesion compared to that seen with freshly isolated eosinophils.

To further verify that $\alpha d\beta 2$ functions as a ligand for VCAM-1, we generated CHO transfectants expressing the human αd and β2 integrin chains and employed them in adhesion assays. These transfected cells expressed αd and β2 integrin chains at modest levels (see Figure 5), and did not express CD11a, CD11b, CD11c, or α4 integrins. As expected, the parental CHO cell line failed to express any of these integrins (data not shown). $\alpha d\beta 2$-transfected CHO cells adhered to VCAM-1-coated wells and adhesion was effectively blocked by an $F(ab')_2$ mAb against the first domain of VCAM-1 as well as by mAbs against either CD18 or αd integrin (Figure 6). In contrast, parental non-transfected CHO cells failed to adhere to VCAM-1, and neither cell type displayed significant adherence to well coated with another adhesion protein, namely, E-selectin (Figure 6 legend).

Discussion

These studies have shown that $\alpha d\beta 2$, like other $\beta 2$ integrins, is expressed on human eosinophils, basophils, and neutrophils. On peripheral blood eosinophils, the level of αd integrin expression is similar to that of $\alpha 4$ integrins, greater than that of CD11c, and less than that of CD11a and CD11b. Stimuli such as PMA and the calcium ionophore A23187 rapidly upregulated eosinophil surface expression of αd integrins, while a more gradual increase in surface expression was seen after 4 to 7 days of culture in media containing IL-5. In adhesion assays, $\alpha d\beta 2$ integrin was shown to function as a ligand for VCAM-1 in both freshly isolated and IL-5-cultured eosinophils; these results were corroborated in adhesion assays employing $\alpha d\beta 2$-transfected CHO cells. Based on mAb blocking studies with freshly isolated eosinophils, adhesion to VCAM-1 was mainly mediated through $\alpha 4$ integrins, the other known ligand for VCAM-1. However, in IL-5-cultured eosinophils, adhesion to VCAM-1 was equally mediated by $\alpha 4$ and αd integrins. Together, these data are the first to demonstrate activation-dependent regulation of $\alpha d\beta 2$ integrin expression and function on human eosinophils and document a novel function for $\alpha d\beta 2$ as an alternative ligand for VCAM-1.

There appear to be preformed stores of αd integrin in eosinophils, as evidenced by the rapid upregulation of surface expression with exposure to PMA or calcium ionophore. These results are similar to those observed for αd integrin and neutrophils (12). The kinetics of enhanced expression with PMA exposure was similar to that of CD11b, suggesting that these two leukointegrins might exist in similar or identical intracellular compartments. The location of this compartment for either integrin in eosinophils is not known; however, in neutrophils, preformed stores of CD11b have been localized to specific granules (27, 28). The immunolocalization of these preformed $\beta 2$ integrin pools, as well as effects of more physiologic activators of eosinophils on integrin expression, is currently under investigation.

In contrast to the rapid mobilization by PMA, a gradual increase in surface αd integrin expression was seen during IL-5 culture. Whether this represents events occurring at the level of transcription or translation, rather than slow mobilization from preformed pools, is not yet known, due in part to difficulties encountered in isolating eosinophil mRNA as well as adverse effects of inhibitors of transcription and translation on eosinophil survival. In examining levels of αd integrin on late phase BAL eosinophils, cells which have already undergone cell adhesion and migration to get to the airway lumen, levels of expression intermediate to those seen on freshly isolated and IL-5 cultured eosinophils were observed. These data suggest that at least a portion of the elevated levels of αd found after IL-5 culture is likely due to increased transcription and translation of αd integrin.

A particularly novel aspect of the present study was the determination that $\alpha d\beta 2$ integrin, expressed on eosinophils and CHO transfectants, can function as a ligand for VCAM-1. While the exact binding site on VCAM-1 is unknown, the finding that a mAb to the $\alpha 4$ integrin binding site in the first domain of VCAM-1 completely blocked $\alpha d\beta 2$ integrin dependent VCAM-1 adhesion strongly suggests that the $\alpha d\beta 2$ binding site is near or identical to that for $\alpha 4$ integrins. Since there is little amino acid homology between αd and $\alpha 4$ integrins, this was unexpected. Whether $\alpha d\beta 2$ integrins can bind to other $\alpha 4$ integrin ligands, such as fibronectin or mucosal addressin cell adhesion molecule-1, is unknown. The finding that $\alpha d\beta 2$ integrin can function as a ligand for VCAM-1 appears to conflict with data presented in the first report on human $\alpha d\beta 2$ integrin by Van der Vieren, et al (12). In this paper it was shown that these same $\alpha d\beta 2$-transfected CHO cells bound to a soluble ICAM-3 construct but not to a VCAM-1-Ig chimeric protein. Possible explanations for this discrepancy include a lower affinity for soluble ligand binding as well as other differences in assays, such as temperature.

Depending on the experimental conditions, both $\alpha 4$ and αd integrins can mediate eosinophil adhesion to VCAM-1. As we have shown in IL-5 cultured eosinophils, the contribution of αd integrin to this adhesion increases in parallel with an increase in its cell surface expression. However, proportionally enhanced αd-mediated adhesion may also be due to an increase in the activation of αd integrins and/or a decrease in the activation state of $\alpha 4$ integrins, since surface levels of $\alpha 4$ integrins remain unchanged and net adhesion decreases after IL-5 culture (Figures 3b and 4b). Another potential paradox from our findings is that although neutrophils express $\alpha d\beta 2$, they do not adhere to 7 domain VCAM-1. The most plausible explanation for this again appears to be related to integrin activation state. Freshly isolated eosinophils and neutrophils express similar levels of αd integrins and, at least for eosinophils, much lower αd integrin dependent adhesion responses as seen compared with activated cells. Whether this can be overcome with neutrophil activation is not yet known.

The function of αd integrin *in vivo* is currently under investigation. Its wide distribution on various tissues, including human bowel wall tissue and synovium, suggests other roles for this integrin (29, 30). In the dog, it was reported that αd integrin is expressed on large granular lymphocytes, where it appears to be a marker of a form of chronic lymphocytic leukemia (31). However, there is no evidence in humans as yet that this leukointegrin is useful as a marker of hematologic disease and, indeed, the distribution of αd integrins on canine peripheral blood leukocytes is different from that seen on human leukocytes. In the rat, αd integrins may play a role in inflammatory diseases, as a monoclonal antibody to αd integrin inhibited the development of IgG immune complex-mediated lung damage (32). In humans, αd integrins may play a role in rheumatoid arthritis, where unusual aggregates of $\alpha d\beta 2$-positive lymphocytes were noted in synovial sublining areas (30). Based on our results, it is plausible that since αd integrins on eosinophils bind to VCAM-1 and can be upregulated with IL-5, this leuko-integrin may play a role in cytokine-primed eosinophil

recruitment to inflammatory sites. Evaluation of this hypothesis, however, will require further investigation.

Figure legends

Figure 1. (a) Expression of β2 (CD18) integrins and α4 (CD49d) integrin on human eosinophils. Representative histograms shown (n=7). (b) Comparison of the surface expression of αd integrin on peripheral blood eosinophils, neutrophils, and basophils. Representative histograms shown ($n \geq 4$).

Figure 2. Expression of both αd integrin (squares) and CD11b (circles) is upregulated rapidly in peripheral blood eosinophils incubated with PMA (50 ng/ml, filled symbols) but not with buffer alone (open symbols). Values are expressed as average MFI ± sem. n=3. Irrelevant isotype control antibody fluorescence (1.7±0.2) was unchanged throughout these experiments. $^*p < 0.05$ for treated versus untreated samples.

Figure 3. (a) Culture of peripheral blood eosinophils for 4 days with IL-5 (10 ng/ml) leads to increased expression of αd but not α4 integrins. Shown are representative histograms from 7 experiments with similar results. (b) Kinetics of changes in surface expression of αd (filled bars) versus α4 (open bars) integrins for eosinophils cultured with IL-5. For comparison, levels on eosinophils obtained from late phase BAL fluid after allergen challenge are also displayed. Values are expressed as net MFI values after subtraction of the irrelevant IgG_1 control MFI values (3.1±0.2, range 0.6-5.7, n=46). $^*p < 0.05$ versus day 0 value, $n \geq 3$.

Figure 4. Adhesion of freshly isolated (panel a, $n \geq 5$) or IL-5 cultured (10 ng/ml x 4-7 days, panel b, $n \geq 3$) eosinophils to immobilized recombinant VCAM-1. Blocking mAbs used included MHM24 (CD11a), clone 44 (CD11b), BU-15 (CD11c), 7E4 (CD18), 240I (αd integrin), and HP2/1 (α4 integrin). Results represent mean ± sem for percent

adhesion; *$p < 0.05$ versus VCAM-1 adhesion without mAb, ‡$p < 0.05$ versus VCAM-1 adhesion in the presence of mAb 240I.

Figure 5. Representative flow cytometry histograms (from 5 experiments with similar results) showing expression of αd and $\beta 2$ integrin chains on transfected CHO cells.

Figure 6. Adhesion of $\alpha d\beta 2$-transfected CHO cells to VCAM-1 is inhibited by mAbs against domain 1 of VCAM-1, αd integrin, and CD18. Adhesion of $\alpha d\beta 2$-transfected CHO cells to VCAM-1 was $16.0 \pm 3.6\%$ (n=11), while their adhesion to E-selectin was $6.1 \pm 3.0\%$ (n=5). Parental non-transfected CHO failed to significantly adhere to either VCAM-1 or E-selectin ($1.9 \pm 0.9\%$ (n=3) and $1.5 \pm 0.5\%$ (n=2), respectively). Values represent mean $\pm$ sem for percent inhibition of adhesion (n=3-4). *$p < 0.0005$ or ‡$p < 0.001$ versus $\alpha d\beta 2$-transfected CHO cell adhesion to VCAM-1.

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Fig. 1a
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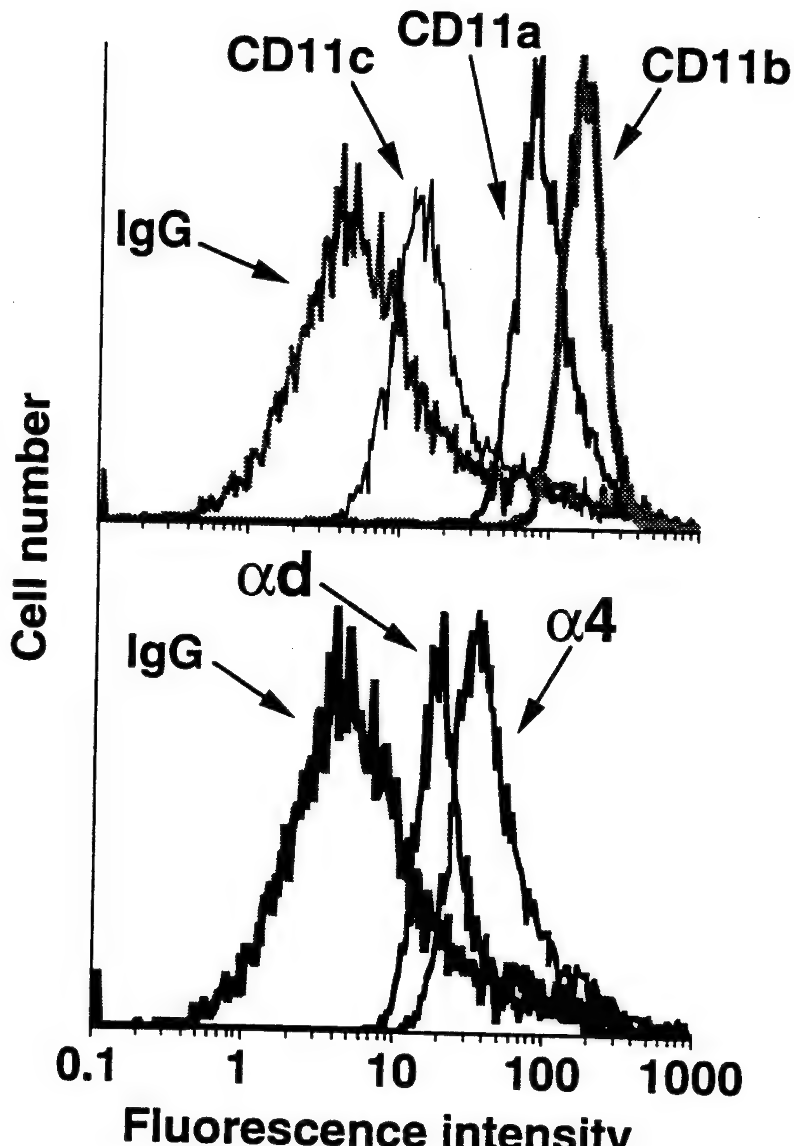
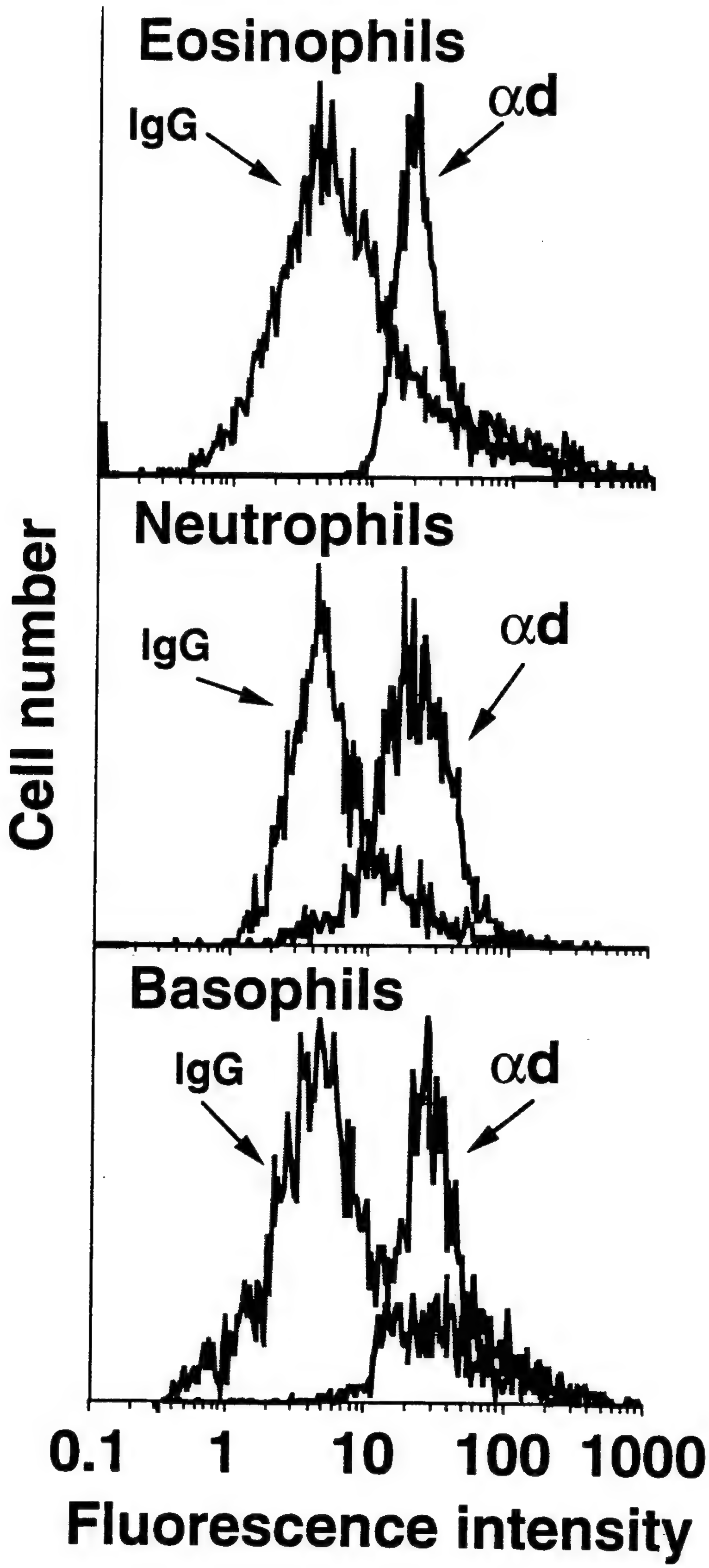
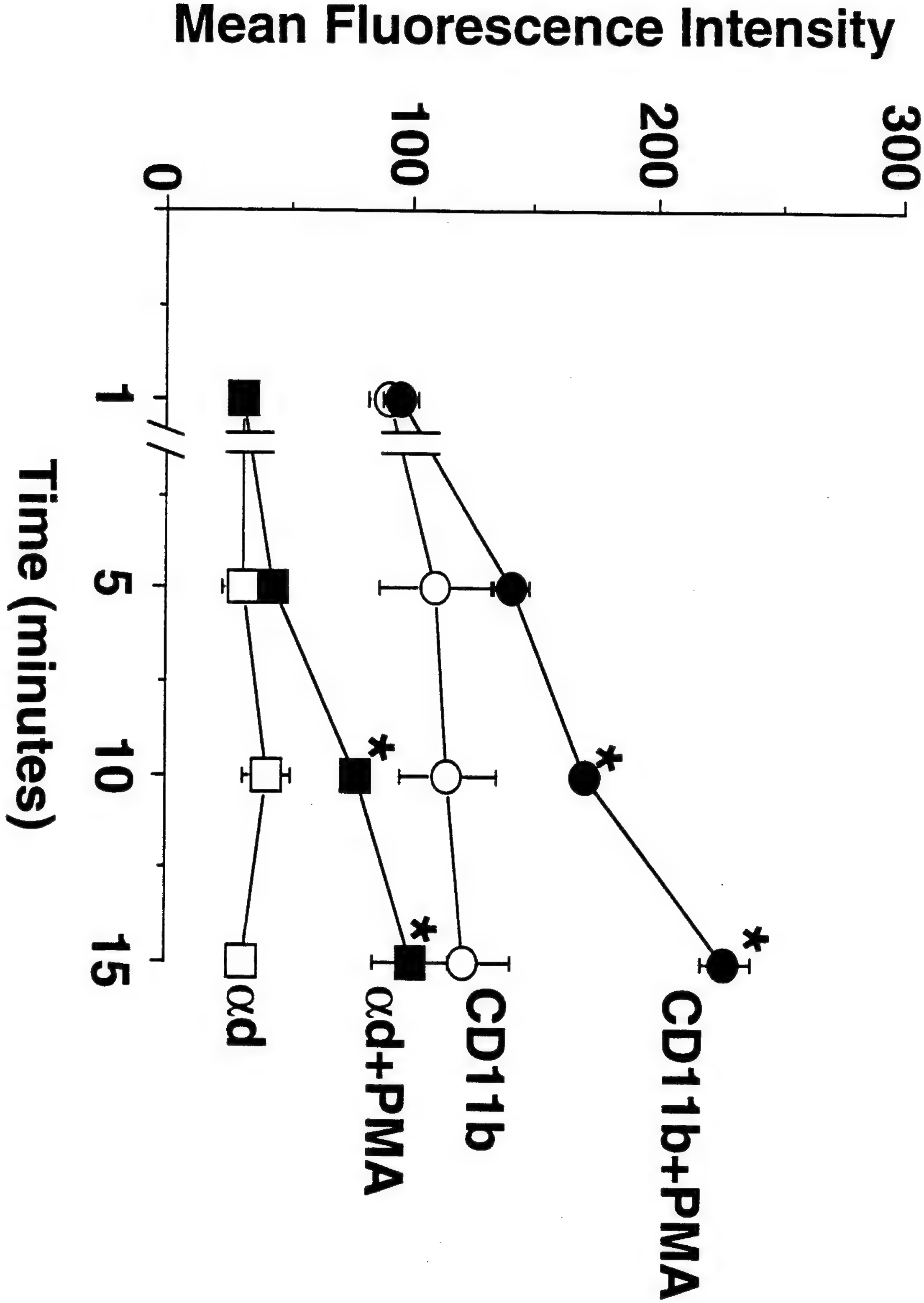


Fig. 1b



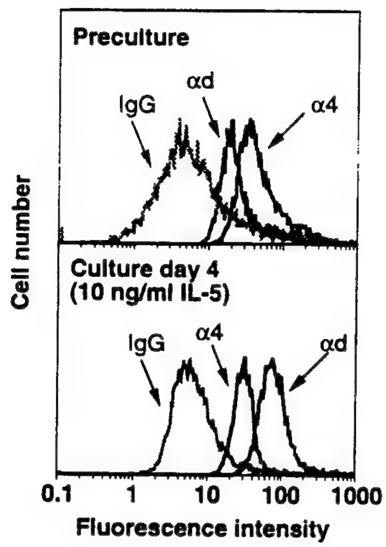
Eosinophils
IgG
αd
Neutrophils
IgG
αd
Basophils
IgG
αd
Cell number
0.1
1
10
100
1000
Fluorescence intensity

Fig. 2



Mean Fluorescence Intensity
0
100
200
300
1
5
10
15
Time (minutes)
CD11b+PMA
CD11b
αd+PMA
αd
*
*
*
*

Fig. 3a


Preculture
IgG
αd
α4
Culture day 4
(10 ng/ml IL-5)
IgG
α4
αd
Cell number
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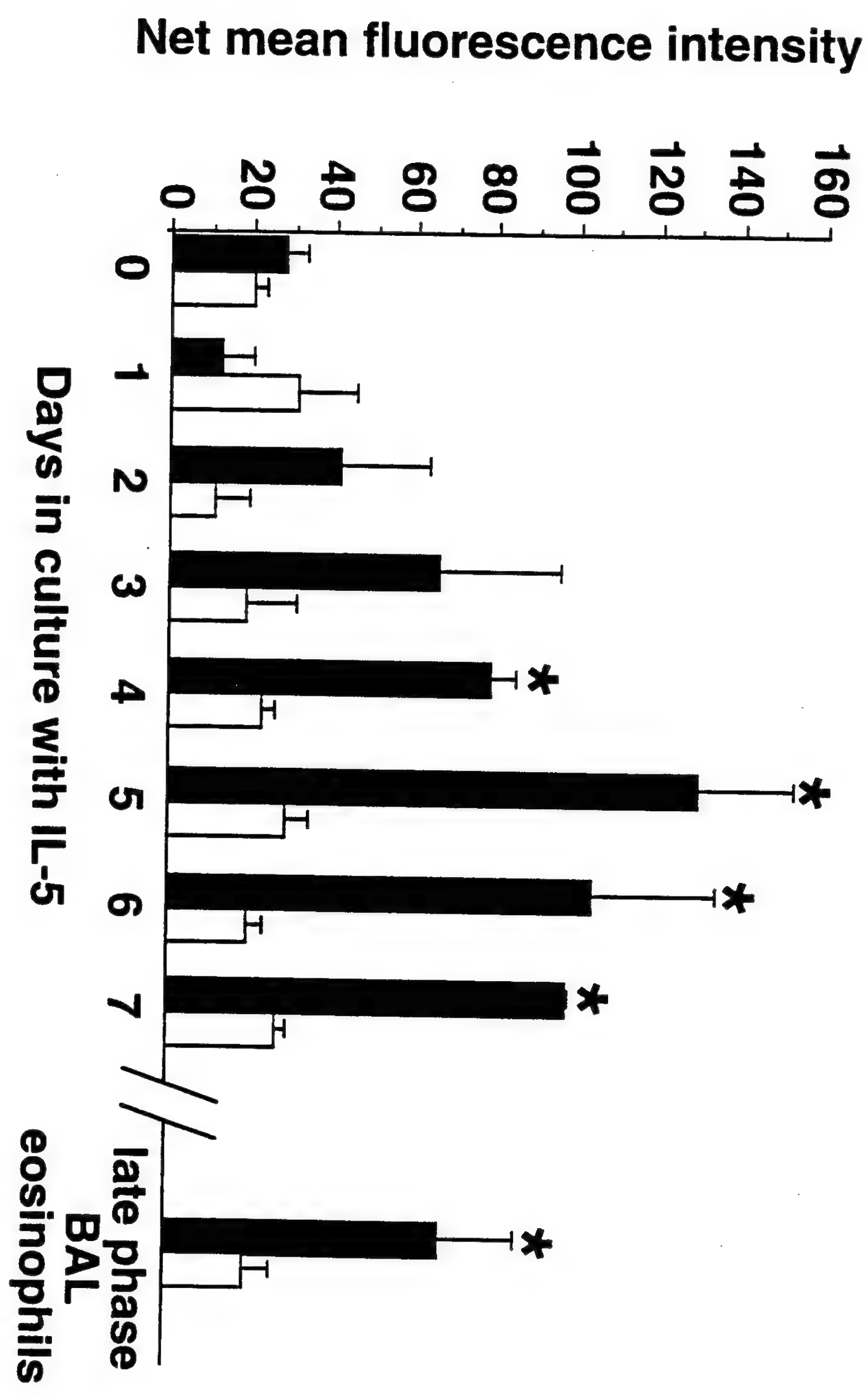
1
10
100
1000
Fluorescence intensity

Fig. 3b



Net mean fluorescence intensity
0
20
40
60
80
100
120
140
160
0
1
2
3
4
5
6
7
late phase
BAL
eosinophils
Days in culture with IL-5

Fig. 4a

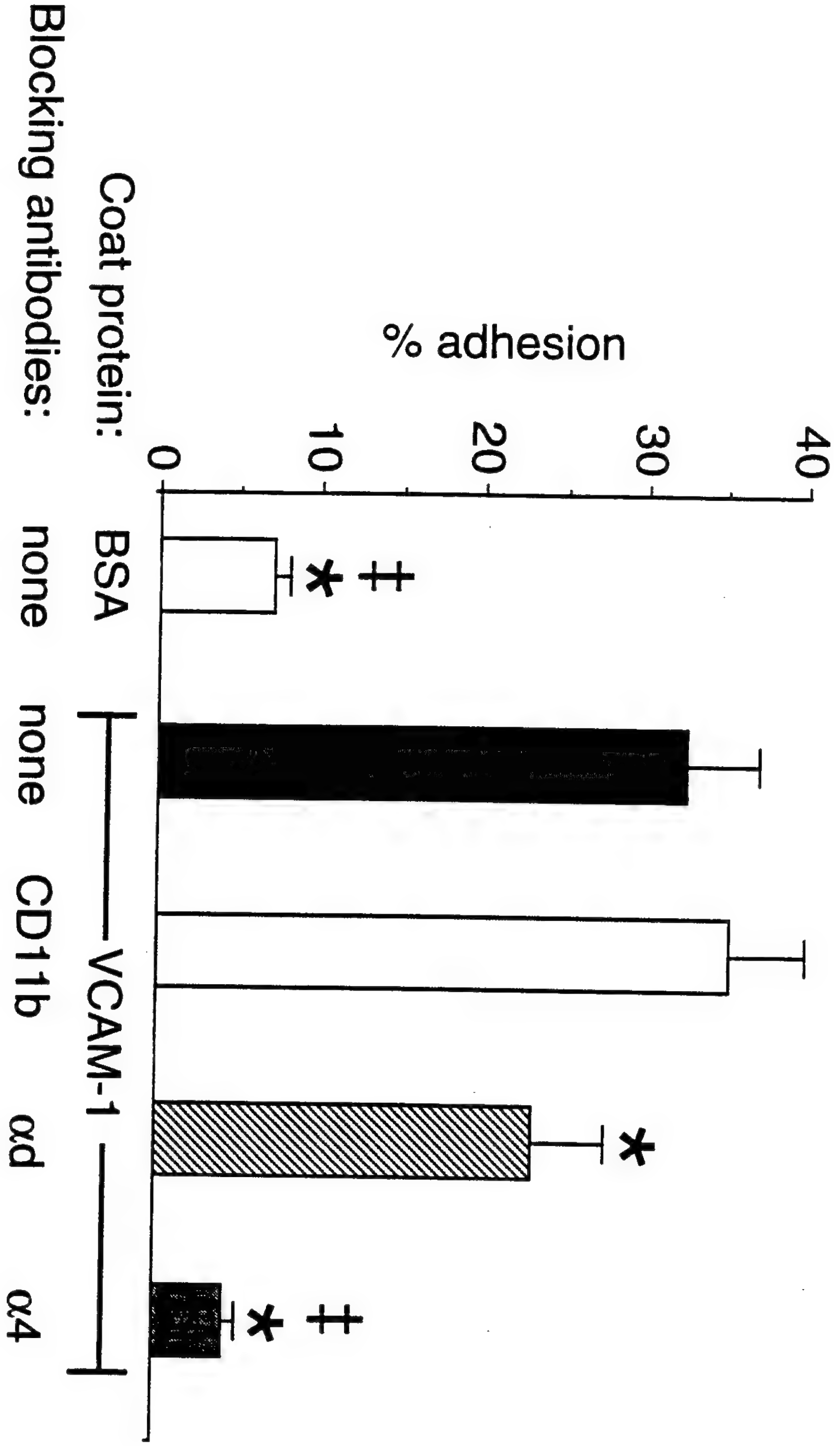
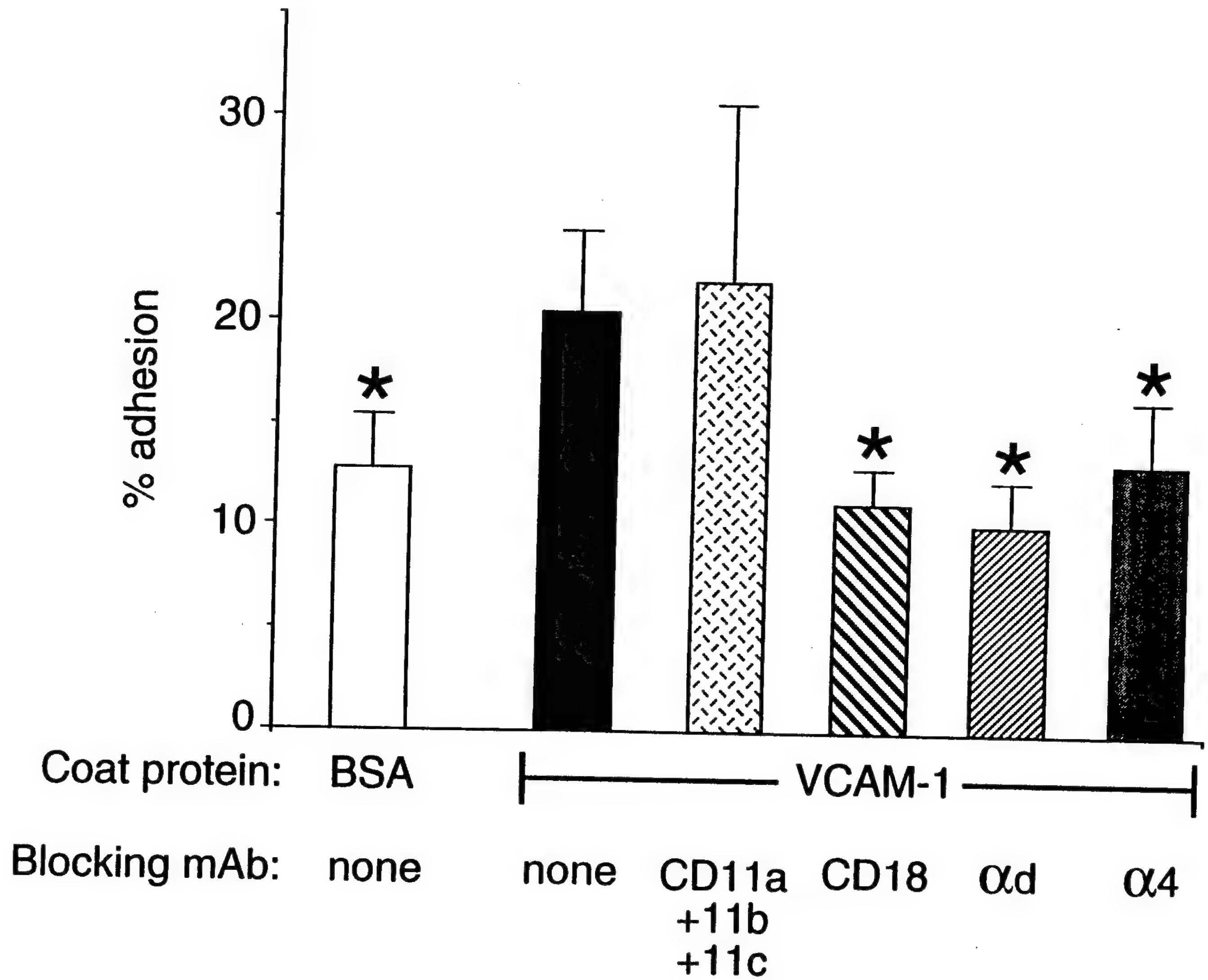

% adhesion
0
10
20
30
40
Coat protein:
Blocking antibodies:
BSA
none
none
CD11b
αd
α4
VCAM-1
*
‡

Fig. 4b



% adhesion
0
10
20
30
*
*
*
*
Coat protein: BSA
VCAM-1
Blocking mAb: none
none
CD11a +11b +11c
CD18
αd
α4

Fig. 5

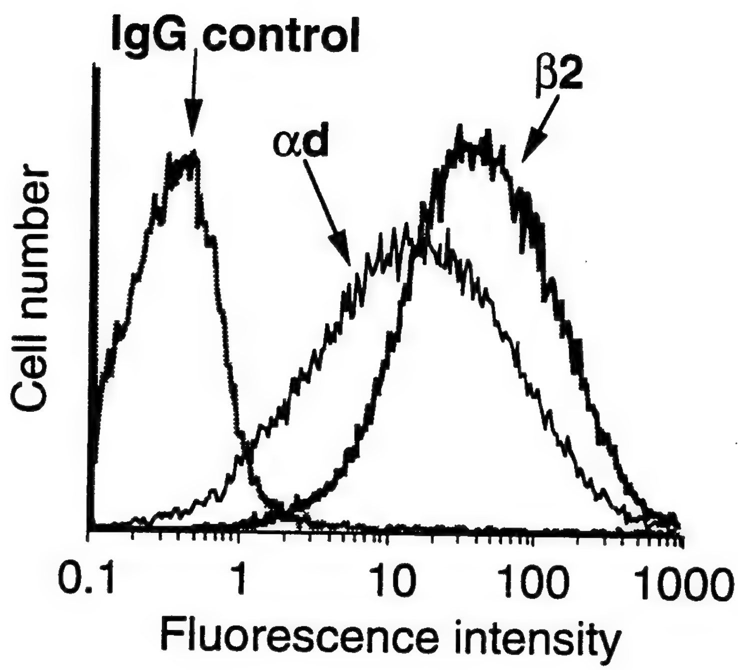
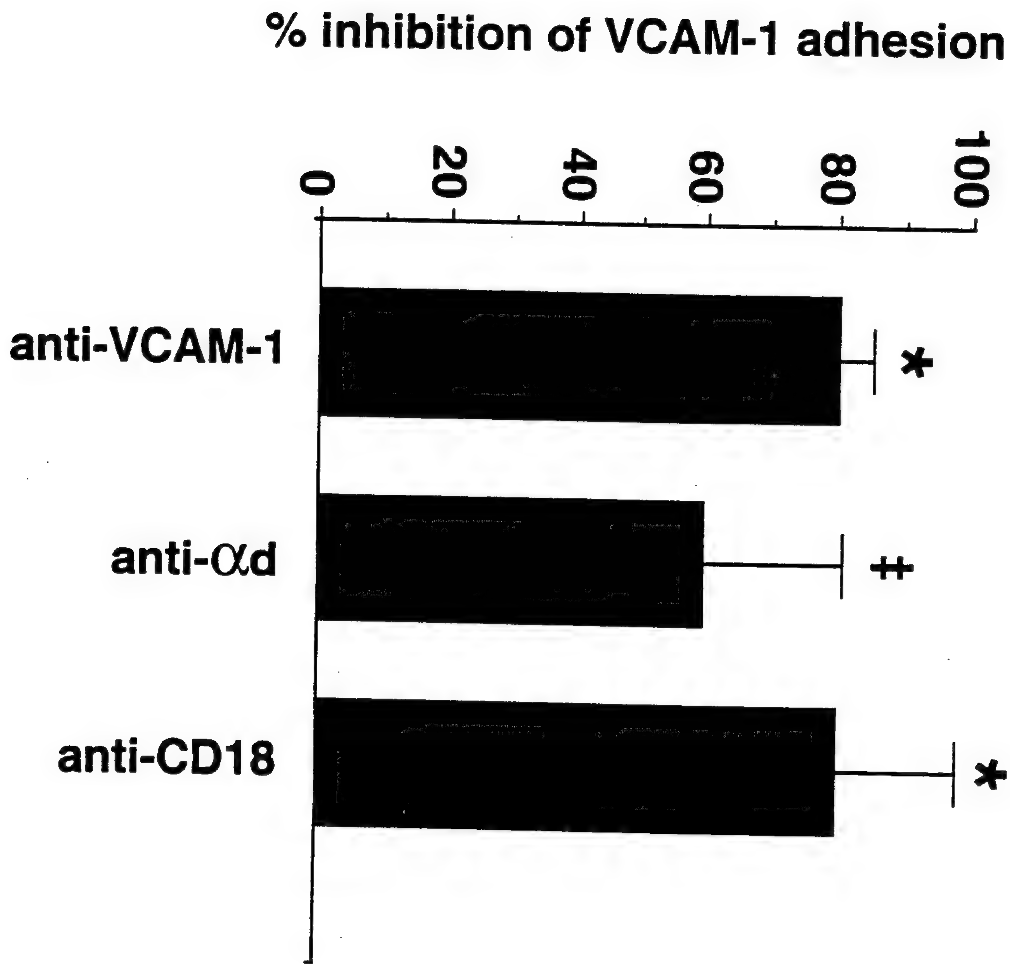

IgG control
β2
αd
Cell number
0.1
1
10
100
1000
Fluorescence intensity

Fig. 6



% inhibition of VCAM-1 adhesion
0
20
40
60
80
100
anti-VCAM-1
anti-αd
anti-CD18
*
‡
*

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6/28/93

Running Title: Steriod regulation of LPS-induced leukocyte rolling and adhesion

Lipopolysaccharide-Induced Leukocyte Rolling and Adhesion in the Rat Mesenteric Microcirculation: Regulation by Glucocorticoids and Role of Cytokines[1]

Kelly L. Davenpeck*, John Zagorski†, Robert P. Schleimer*, and Bruce S. Bochner[2]*

From the *Department of Medicine, Division of Clinical Immunology, The Johns Hopkins University School of Medicine, Baltimore MD 21224, and † Laboratory of Immunology, National Institute of Dental Research, Bethesda MD 20892

Key Words: lipopolysaccharide, adhesion molecules, cytokines, in vivo animal model, immunomodulators

Abstract

A common side effect of high dose glucocorticoid therapy is increased susceptibility to bacterial infection, an effect which is in part mediated through inhibition of leukocyte recruitment to infected areas. However, the sites at which glucocorticoids act to prevent the multistep process of leukocyte recruitment have not been fully established. In this study, the effects of the glucocorticoid dexamethasone (DEX) on leukocyte-endothelial interactions, in response to bacterial lipopolysaccharide (LPS) were examined utilizing a model of rat mesenteric intravital microscopy. Pretreatment of rats with DEX (0.5 mg/kg) for 18 hrs or 30 min prior to stimulation with LPS significantly inhibited LPS-induced leukocyte rolling and adhesion in mesenteric postcapillary venules. Pretreatment with DEX also inhibited LPS-induced changes in expression of L-selectin and a shared epitope of CD11b/c on circulating neutrophils. These effects of DEX may be due to DEX inhibition of IL-1, TNF and cytokine-induced neutrophil chemoattractant-1 (CINC-1)[3] generation, as antagonists to these mediators were able to mimic DEX effects on leukocyte-endothelial interactions and circulating leukocyte phenotype. These data indicate that inhibition of cytokine- and chemokine-induced leukocyte-endothelial interactions may be a primary mechanism by which glucocorticoids inhibit leukocyte recruitment to bacterial agents and thus increase susceptibility to infection.

Introduction

Glucocorticoids have potent immunosuppressive effects and are widely used in the management of chronic inflammatory diseases. Despite their therapeutic benefits, glucocorticoid excess results in a variety of side effects, including enhanced susceptibility to bacterial infection. One of the primary mechanisms by which glucocorticoids are thought to suppress the body's response to bacterial infection is through inhibition of leukocyte recruitment to infected areas (1). Leukocytes play a crucial role in the destruction of opportunistic and pathogenic organisms, and movement of leukocytes out of the circulation into infected tissues is essential for bacterial killing to occur. Bacterial lipopolysaccharide (LPS), a component of the outer wall of most Gram-negative bacteria, is a potent inflammatory agent and plays a primary role in bacterial-induced leukocyte recruitment (2-4). Though glucocorticoids have been demonstrated to inhibit LPS-induced leukocyte recruitment (1, 5, 6), the sites at which glucocorticoids act to prevent the multistep-cascade of leukocyte recruitment have not been fully defined.

Recent data concerning the important role of leukocyte and endothelial adhesion molecules in leukocyte recruitment has led to the speculation that glucocorticoid-mediated inhibition of the inflammatory response, and in particular leukocyte recruitment, may be the result of alterations in the expression and/or function of the leukocyte and endothelial adhesion molecules which mediate leukocyte extravasation. In vitro studies examining the direct effect of glucocorticoids on adhesion molecule expression have not yielded definitive data. For instance, Kaiser et al. found that the glucocorticoid budesonide did not inhibit IL-1 or TNFα-induced expression of E-selectin, intracellular adhesion molecule-1, or vascular cell adhesion molecule-1 on human umbilical vein endothelial cells (7), while Cronstein et al. found dexamethasone to be effective in inhibiting both LPS- and IL-1-induced synthesis and expression of E-selectin and intracellular adhesion molecule-1 (8). Evidence concerning the effects of glucocorticoids on leukocyte adhesion molecule expression are equally inconclusive. Schleimer et al. (9) reported no effect of glucocorticoids on human neutrophil adhesion responses, while in vivo studies have described changes in expression of leukocyte adhesion molecules, particularly ß2 integrins and

L-selectin, following administration of glucocorticoids (5, 10, 11). Thus, whether glucocorticoids inhibit leukocyte recruitment to sites of inflammation by directly altering adhesion molecule expression remains unresolved.

An alternate and perhaps more likely mechanism by which glucocorticoids may inhibit leukocyte recruitment in response to LPS is through inhibition of inflammatory mediator production and/or release, an effect which could indirectly alter adhesion molecule expression (12). LPS is a potent stimulus for cytokine and chemokine release from several cell types including monocytes, macrophages, and endothelial cells. In vivo, cytokines such as IL-1 and TNFα are rapidly released in response to LPS (13-15) and both of these cytokines induce endothelial adhesion molecule expression (7). Glucocorticoids inhibit production of these cytokines (12, 16, 17), as well as chemokines of the C-X-C family such as CINC, which is involved in mediating leukocyte recruitment in response to LPS (18-20). Thus, glucocorticoids may inhibit leukocyte recruitment by inhibiting the mediators which induce adhesion molecule-mediated leukocyte endothelial interactions and leukocyte migration.

We have previously demonstrated that superfusion of a single loop of rat mesentery with LPS results in dose- and time-dependent increases in leukocyte rolling and adherence in mesenteric postcapillary venules, and that LPS-induced changes in leukocyte rolling and adhesion are largely mediated by both P- and L-selectin (21). The major aims of the experiments described herein were to: 1) determine if glucocorticoids block leukocyte recruitment in response to LPS by inhibiting adhesion molecule-mediated leukocyte-endothelial interactions; and 2) to determine if glucocorticoids affect leukocyte-endothelial interactions through inhibition of the release or actions of cytokines and/or chemokines. To achieve the first aim we utilized an established in vivo model of rat intravital microscopy to directly examine the effects of the glucocorticoid dexamethasone (DEX) on LPS-induced leukocyte rolling along, and adhesion to, the vascular endothelium. To address the second aim we examined whether antagonists to the cytokines IL-1 and TNF, or to the chemokine cytokine-induced neutrophil chemoattractant-1 (CINC-1), given alone or in combination, could mimic the effects of glucocorticoids on LPS-

induced leukocyte-endothelial interactions. We demonstrate that either prolonged (i.e., 18 hours) or short term (i.e., 30 min) pretreatment of rats with DEX significantly inhibited the selectin-mediated leukocyte rolling and adhesion induced by superfusion of the mesentery with LPS and prevented LPS-induced alterations in circulating leukocyte adhesion molecule expression. Antagonism of IL-1, TNF and CINC-1 inhibited LPS-induced leukocyte rolling and adhesion in a manner similar to DEX treatment, thus supporting the hypothesis that glucocorticoid suppression of leukocyte recruitment to LPS is mediate through effects on cytokine generation and/or release.

Materials and Methods

Rat Mesenteric Intravital Microscopy: In accordance with an animal research protocol approved by the Johns Hopkins University Animal Care and Use Committee, male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) underwent anesthesia and surgical manipulation with exteriorization of ileal mesentery to facilitate video intravital microscopy, as previously described (21). The ileum and mesentery were superfused throughout the experiment with a modified Krebs-Henseleit solution (in mM:118 NaCl, 4.74 KCl, 2.45 $CaCl_2$, 1.19 KH_2PO_4, 1.19 $MgSO_4$, 12.5 $NaHCO_3$) (Sigma Chemical Co., St. Louis, MO) heated to 37°C and bubbled with 95% N_2 and 5% CO_2. A Zeiss Axioskop fixed stage upright microscope was used for observation of the mesenteric microcirculation. The image was projected by a high resolution CCD camera (Hamamatsu, Japan) to a black and white high resolution monitor and the image recorded with a videocassette recorder (Sony Corp. of America, Park Ridge, NJ). Red blood cell velocity was determined on-line using an optical Doppler velocimeter (22) (Microcirculation Research Institute, College Station, TX).

Mean venular diameter, numbers of rolling and numbers of adherent leukocytes were determined off-line by play-back of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity slower than that of red cells. The rolling rate (i.e. leukocyte flux) was expressed as the number of cells moving past a fixed point per minute. A leukocyte was determined to be adherent if it remained stationary for > 30 sec. Adherence was expressed as number of leukocytes/100 µm of vessel. Venular wall shear rate (γ) was calculated based on red blood cell velocity and venular diameter using the formula $\gamma = 8$ (Vmean/D), where Vmean is the mean red blood cell velocity (i.e., center line velocity/1.6) and "D" is mean venular diameter (23).

Experimental Protocol. Following exteriorization and placement of a loop of ileal mesentery in the superfusion chamber, a 23-42 µm diameter post-capillary venule was chosen for observation. A baseline control recording of 2 min duration was made, and the tissue was then allowed to stabilize for 30 min. If leukocyte rolling or adhesion was observed to increase during

this period the experiment was terminated. Following the 30 min stabilization period. a second video recording (time 0) was made to establish basal values for leukocyte rolling and adherence. and leukocyte rolling velocities. To minimize the influence of pre-activation of the tissue. only vessels in which leukocyte rolling was ≤ 30 cells/min and adhesion ≤ 3 cells/100 μm of venular endothelium were utilized for study.

For some experiments. rats were pretreated either 18 hours or 30 min prior to LPS superfusion with dexamethasone-21-phosphate (DEX) (Sigma) at a dose of 0.5 mg/kg given as a subcutaneous injection in 300 μl of sterile phosphate buffered saline (PBS). Control rats were pretreated 18 hours or 30 min before LPS superfusion with subcutaneous injections of either 300 μl of PBS alone or with testosterone given at a dose of 0.5 mg/kg (n =4 for 18 hr pretreatment and n = 2 for 30 min pretreatment: data are combined for n = 6 because a similar lack of effect was seen. see results). To examine the effects of DEX pretreatment on basal or unstimulated leukocyte rolling and adhesion. a group of rats was pretreated with dexamethasone (i.e.. 18 hours or 30 min) and their mesentery was superfused with Krebs-Henseleit buffer alone for the entire 2 hour period. No significant differences in unstimulated leukocyte rolling or adhesion were seen with either 18 hr (n = 4) or 30 min (n = 2) DEX pretreatment when compared to rats not treated with DEX (21). The data from these two groups (i.e.. 18 hours or 30 min DEX pretreatment) were combined (n = 6) and together referred to as "buffer control". Soluble murine IL-1 receptor (IL-1R. 100 μg/kg) (24) and/or soluble human TNF receptor linked to the Fc region of human IgG1 (TNFR:Fc. 100 μg/kg) (25). generously provided by Immunex Corporation (Seattle. WA). were given. alone or simultaneously. ten min prior to LPS superfusion and again following 60 min of superfusion. In other experiments. a neutralizing anti-CINC-1 polyclonal antibody (19) was given intravenously (2 mg/rat) ten minutes prior to LPS superfusion.

The mesentery was superfused with 1 μg/ml of lipopolysaccharide (LPS. from Escherichia coli serotype 0127:B8. Sigma Chemical Co. St. Louis. MO. Lot 63H4010) in modified Krebs-Henseleit solution for 120 min as previously described (21). This concentration was shown to be optimal in our previous studies and induced similar effects to those seen with

LPS from other bacterial serotypes (21). LPS superfusion was initiated immediately following the 0 minute video recording, and then subsequent 2 minute recordings were made at 30. 60. 90. and 120 min after initiation of superfusion for determination of leukocyte rolling and adherence. and leukocyte rolling velocity. Arterial blood samples (<100 µl) were obtained at each of the above time points and circulating total white blood cell (WBC) numbers determined by light microscopic counting (Unopette. Test 5856, Becton-Dickinson. Rutherford. NJ). as described (21). Whole blood smears for determination of leukocyte differentials were also made at baseline. 0, and 120 min. Cell differentials were determined by Diff-Quick staining (Shandon. Pittsburgh, PA). In some animals. arterial blood samples (1 ml) were taken prior to the initial video recording and again after two hours. and leukocytes were isolated for flow cytometric analysis of leukocyte adhesion molecule expression (26).

Rat Leukocyte Isolation and Flow Cytometric Analysis of Leukocyte Adhesion Molecules. Murine-anti-rat CD11a (WT.1, IgG2a. 5 µg/ml), CD11b/c (OX-42. IgG2a. 1 µg/ml). and CD18 (WT.3. IgG1. 5 µg/ml) (Pharmingen. San Diego, CA) and the α4 integrin mAb TA-2 (IgG1. 1 µg/ml) (Seikagaku America. Inc.. Rockville. MD) were purchased and used at the indicated saturating concentrations. A murine anti-rat L-selectin mAb (LAM1-116. IgG1. 3 µg/ml) was generously provided by Drs. Thomas Tedder and Douglas Steeber (Duke University Medical Center. Durham NC). Control non-binding mouse IgG1 (10 µg/ml) and IgG2a (10 µg/ml) were obtained from Coulter Corporation (Hialeah. FL). Labeling of cells for indirect immunofluorescence was performed essentially as described (26. 27) using saturating concentrations of FITC-conjugated goat-anti-mouse secondary antibody (Caltag Laboratories. South San Francisco. CA). Cells were immediately analyzed without fixation using an EPICS Profile II Flow cytometer (Coulter Corporation). Isotype control staining typically yielded values for mean fluorescence of 1-3.

To determine the effects of local LPS superfusion on circulating leukocytes. leukocyte adhesion molecule expression was examined on isolated leukocytes from animals undergoing 2

hour superfusion of a single loop of mesentery with LPS (i.e., application of LPS for intravital microscopy) and compared to adhesion molecule expression on leukocytes from buffer control animals. All animals underwent the same surgical procedures as described for intravital microscopy and the mesentery was secured for intravital microscopic observation as described above. An arterial blood sample (1 ml) was taken, placed in EDTA, and stored at 4°C prior to the initial video recording and the blood volume replaced with normal saline. Following superfusion of the mesentery with LPS or normal Krebs for 2 hours, a second blood sample was obtained. Mixed populations of whole blood leukocytes were isolated from EDTA-anticoagulated arterial blood samples. A leukocyte-rich buffy coat was obtained by centrifugation at 400g for 20 min at 22°C, and contaminating red blood cells were removed via hypotonic lysis performed at 4°C. Cell differentials were determined by Diff-Quik staining (Shandon, Pittsburgh, PA) and viability was confirmed by erythrosin B dye exclusion. Leukocyte adhesion molecule expression was examined for all groups of rats including LPS + vehicle, DEX pretreated (18 hours and 30 min) + LPS, DEX treated buffer controls, IL-1R/TNFR:Fc treated + LPS, and anti-CINC-1 antibody + LPS.

The ability of LPS and other leukocyte stimulatory agents to alter leukocyte adhesion molecule expression in vitro following in vivo treatment with DEX was also examined. Heparin-anticoagulated whole blood was obtained from animals treated with vehicle (300 µl PBS), 18 hour DEX treated (0.5 mg/kg) or 30 min DEX (0.5 mg/kg). LPS (1 - 1000 µg/ml) was added to aliquots of whole blood which were then incubated for 30 min at 37°C. Contaminating red blood cells were then removed by hypotonic lysis, and ß2 integrin and L-selectin expression was examined via flow cytometry as above.

Data Analysis. All data are presented as mean ± SEM. Data were compared by analysis of variance (ANOVA) using post-hoc analysis with Fischer's corrected t-test. Probabilities of 0.05 or less were considered statistically significant.

Results

Prolonged and short term DEX pretreatment blocks LPS-induced leukocyte-endothelial interaction. Superfusion of the rat mesentery with 1 µg/ml of LPS resulted in a rapid and significant increase in leukocyte rolling and adhesion as compared to buffer control animals (Figure 1 and (21)). Increases in both rolling (panel A) and adhesion (panel B) were significant by 30 min. continued to increase by 60 min. and were maintained for at least 120 min.

To determine if glucocorticoids could inhibit LPS-induced leukocyte recruitment by altering leukocyte-endothelial interactions. rats were pretreated with DEX for either 18 hrs or 30 min prior to initiation of LPS superfusion and effects on leukocyte rolling and adhesion were quantified. DEX pretreatment of rats for 18 hours completely inhibited LPS-induced changes in leukocyte rolling and adhesion (Figure 1 A & B). Values for leukocyte rolling and adhesion in 18 hr DEX pretreated animals in which the mesentery was superfused with LPS were not significantly different than values from buffer control animals. Although initially intended as a control condition. pretreatment of rats with DEX for only 30 min prior to initiation of LPS superfusion also significantly attenuated leukocyte rolling, and completely inhibited leukocyte adhesion (Figure 1 A & B). Inhibition of leukocyte rolling was not complete. as was seen with 18 hour pretreatment. but there was >70% inhibition of leukocyte rolling at all time points. Inhibition of LPS-induced leukocyte rolling and adhesion appears to be specific to glucocorticoids. as pretreatment of rats with the sex steroid testosterone (0.5 mg/kg). either 18 hrs or 30 min prior to LPS superfusion. had no effect on LPS-induced leukocyte rolling and adhesion (Figure 1 A & B).

DEX effects are not mediated by changes in hemodynamic parameters or circulating leukocyte populations. We have previously shown that the LPS-induced changes in leukocyte rolling and adhesion observed in this model system occurred in the absence of significant changes in venular wall shear rate (21). As pretreatment of rats with DEX occurred prior to set-up and selection of mesenteric venules for observation by intravital microscopy, potential effects of DEX on venular

diameter and red cell velocity in mesenteric postcapillary venules cannot be excluded. However, in an attempt to insure that hemodynamic parameters did not contribute to potential changes in leukocyte rolling and adhesion, vessels within the same diameter range (25 to 40 µm) and with similar red cell velocities to those utilized in non-DEX treated animals were selectively chosen for observation. Thus, there was no significant difference in venular wall shear rates among the various treatment groups (Table 1) and under these conditions DEX effects can not be attributed to shear-related effects.

To determine if changes in the number or differential of circulating leukocytes was responsible for the DEX-mediated decreases in LPS-induced leukocyte rolling and adhesion, we monitored these parameters. As reported previously (21), circulating leukocyte numbers increased in both buffer control and LPS-treated animals (Table 1). In rats pretreated with DEX for 18 hours (i.e., buffer control and DEX + LPS), circulating leukocyte numbers at baseline were decreased compared to non-DEX treated rats, although these values did not reach statistical significance when examined utilizing ANOVA. Despite the decreased number of circulating leukocytes at baseline, circulating leukocyte counts increased in these animals in a manner similar to that was observed in non-DEX treated rats. Administration of DEX 30 min prior to LPS superfusion had no effect on circulating leukocyte numbers at baseline or at any of the later time points. Similarly, administration of testosterone had no effects on circulating leukocyte numbers at any of the time points examined (Table 1).

Because glucocorticoids can alter circulating leukocyte populations, we also examined leukocyte differentials at baseline and at the termination of the intravital microscopy protocol. Under baseline control conditions, the majority (~ 80%) of circulating leukocytes in the rat are lymphocytes, with neutrophils making up approximately 10-20% and the remainder monocytes and eosinophils (Figure 2). Following surgical manipulation and intravital microscopy the leukocyte differential is changed substantially, with neutrophils making up the majority of circulating cells (~ 60%) (Figure 2). Similar to changes in circulating leukocyte numbers, this change in leukocyte differential occurs both in the presence and absence of LPS (i.e., buffer

control) (21), implying that it is not a direct effect of LPS superfusion of the mesentery. Pretreatment of rats with DEX for 18 hours resulted in changes in circulating leukocyte differentials as compared to non-DEX treated rats (Figure 2). When rats were pretreated with DEX for 18 hrs. there was a significant decrease in the percentage of circulating lymphocytes. with a concomitant increase in the percentage of neutrophils. Following surgical manipulation and 2 hr LPS superfusion. rats pretreated with DEX for 18 hrs continued to have a significantly increased percentage of circulating neutrophils. and decreased percentage of lymphocytes. when compared to non-DEX treated rats. This was not seen in rats pretreated with DEX for only 30 min. Despite the significant increase in the percentage of circulating neutrophils. rats treated with DEX for 18 hrs had the lowest number of rolling and adherent cells.

DEX inhibits LPS-induced changes in L-selectin and β2 integrin expression on circulating neutrophils. As DEX was demonstrated to significantly decrease leukocyte rolling and adhesion. a series of experiment was performed to determine if DEX was inhibiting leukocyte-endothelial interactions by altering the expression of L-selectin and/or the ß2 integrins on circulating neutrophils. To examine this. mixed leukocyte populations were obtained from whole blood samples taken at baseline (after surgical manipulation) and after 2 hrs of LPS superfusion: expression of various adhesion molecules was examined by indirect immunofluorescence and flow cytometry. In the first series of experiments. the effect of LPS superfusion on circulating leukocyte phenotype was examined with or without DEX pretreatment. Superfusion with LPS for 2 hrs significantly altered the phenotype of circulating leukocytes. In particular. LPS superfusion resulted in a significant decrease in the percentage of neutrophils expressing L-selectin (Figure 3A), although lymphocyte L-selectin expression was not altered (data not shown). The decrease in neutrophil L-selectin expression was accompanied by an upregulation of the expression of a shared CD11b/CD11c epitope (Figure 3B). However. expression of the ß2 integrin subunit CD18. as well as CD11a and α4 integrin. were not altered by LPS superfusion (data not shown). The effects of DEX on this response are also shown in Figure 3. Both 18 hr

and 30 min pretreatment with DEX completely inhibited the changes in leukocyte adhesion molecule expression brought about by LPS superfusion of the mesentery (Figure 3 A & B). In contrast, no significant changes in leukocyte L-selectin or ß2 integrin expression were observed in control buffer-superfused animals (Figure 3 A & B). These data indicate that, unlike changes in circulating leukocyte numbers and differentials, changes in circulating leukocyte phenotype are a direct result of LPS superfusion of the mesentery (i.e., not due to anesthesia or surgical manipulation), and are completely inhibitable by DEX.

To determine if DEX prevented LPS-induced L-selectin shedding and upregulation of the CD11b/c integrins in vivo by altering the ability of leukocytes to respond to LPS, we next performed a series of experiments in which whole blood was obtained from control and DEX treated rats and stimulated ex vivo with LPS. Flow cytometric analysis of changes in leukocyte phenotype was then performed. Stimulation of whole blood obtained from control and DEX (18 hrs and 30 min) treated animals with LPS (1 to 1000 ng/ml) for 30 min at 37°C resulted in concentration-dependent shedding of L-selectin from rat neutrophils (Figure 3C). Pretreatment with DEX for 18 hrs significantly enhanced ex vivo LPS-induced L-selectin shedding, resulting in a log fold reduction in the concentration of LPS required for this effect. In contrast, ex vivo stimulation of rat neutrophils with LPS did not result in any significant change in ß2 integrin expression when neutrophils were obtained from either control or DEX treated rats (data not shown). These data clearly indicate that DEX does not inhibit neutrophil responsiveness to LPS, but also indicate that the changes in adhesion molecule expression in vivo may not be a direct result of neutrophil stimulation with LPS.

Role for IL-1 and TNF in LPS-induced leukocyte rolling and adhesion. Having determined that the ability of DEX to inhibit LPS-induced leukocyte-endothelial interactions was not the result of direct effects of DEX on LPS-induced changes in leukocyte adhesion molecule expression, we performed a series of experiments aimed at examining whether DEX effects on LPS-induced leukocyte rolling and adhesion were mediated indirectly, by inhibition of inflammatory

mediators. Because glucocorticoids are potent inhibitors of cytokine production, and LPS induces production of many cytokines including IL-1 and TNF, we performed experiments utilizing soluble IL-1 (IL-1R) and TNF (TNFR:Fc) receptors to determine if these cytokines were involved in mediating LPS-induced leukocyte rolling and adhesion. Administration of either IL-1R or TNFR:Fc resulted in partial inhibition of leukocyte rolling, although there was a different time course of inhibition for each soluble receptor (Figure 4A). Soluble IL-1R alone did not inhibit early leukocyte rolling (30 and 60 min), but significantly inhibited leukocyte rolling at later time points (90 and 120 min). Conversely, TNFR:Fc inhibited leukocyte rolling at all time points, though inhibition was most pronounced at the earliest time point (30 min). Both antagonist had significant effects on leukocyte rolling when given alone, but the greatest inhibition of leukocyte rolling was observed when IL-1R and TNFR:Fc were given together (Figure 4A). IL-1R and TNFR:Fc together significantly decreased leukocyte rolling at all time points, and values for rolling were not significantly different from values in DEX treated rats. Similar results were found for leukocyte adhesion (Figure 4 B). Administration of either soluble receptor alone resulted in partial inhibition of leukocyte adhesion, while IL-1R and TNFR:Fc given together completely inhibited adhesion at all time points (Figure 4 B). Together, these data demonstrate that both IL-1 and TNF play an integral role in LPS-induced leukocyte endothelial-interactions, and are consistent with the hypothesis that DEX inhibits LPS-induced rolling and adhesion by inhibiting production of these cytokines.

Role for cytokine-induced neutrophil chemoattractant-1 (CINC-1) in LPS-induced leukocyte rolling and adhesion. Interleukin-1, TNF and LPS have all been demonstrated to result in production and release of the neutrophil active chemokine CINC-1 in the rat, and CINC-1 has been demonstrated to play a significant role in LPS-induced leukocyte recruitment (18-20). To determine if CINC-1, induced by LPS or the cytokines IL-1 or TNF, was playing a role in leukocyte-endothelial interaction in our model, a polyclonal antibody directed to CINC-1 was administered (2 mg/rat) ten minutes prior to superfusion of the mesentery with LPS.

Administration of the CINC-1 polyclonal antibody had no effect on early (30 min) leukocyte rolling or adhesion (Figure 5 A & B). However, leukocyte rolling was significantly attenuated by 60 min and remained depressed throughout the remainder of the superfusion time (Figure 5 A). Leukocyte adhesion was also decreased after 60 min, and these values reached statistical significance at 60, 90, and 120 min (Figure 5 B). Since IL-1R and TNFR:Fc given together completely blocked leukocyte rolling and adhesion at the earliest time point (30 min), and anti-CINC antiboby had no effect at this time point, it is possible that production of CINC-1 is downstream of cytokine production and thus may be induced by IL-1 and/or TNF. This is further supported by the finding that values for leukocyte rolling and adhesion obtained from rats pretreated with all three antagonists (i.e., IL-1R, TNFR:Fc, and anti-CINC-1 antibody) simultaneously were not significantly different from values obtained when only the cytokine antagonists (IL-1R and TNFR:Fc) were given (data not shown).

Changes in LPS-induced leukocyte rolling and adhesion observed with administration of IL-1R or TNFR:Fc either alone or in combination, or with administration of anti-CINC-1 antibody were not the result of hemodynamic changes. Neither IL-1R, TNFR:Fc, nor anti-CINC-1 antibody had any effect on venular diameter, RBC velocity or venular wall shear rates (data not shown).

Role for cytokines and chemokines in systemic changes in circulating leukocyte numbers and phenotype. As IL-1, TNF and CINC-1 were all demonstrated to play a role in LPS-induced changes in leukocyte rolling and adhesion, experiments were performed to determine if these inflammatory mediators were playing a role in in vivo changes in circulating leukocyte phenotype observed with LPS superfusion of the mesentery. Similar to experiments described earlier, mixed leukocyte populations were isolated at baseline and after LPS superfusion from animals given either the IL-1R and TNFR:Fc combination or the CINC-1 polyclonal antibody. Expression of L-selectin and the ß2 integrins was analyzed by flow cytometry. Similar to effects of DEX, administration of both IL-1R and TNFR:Fc in combination, or the anti-CINC-1

antibody, significantly attenuated the LPS-induced decreases in neutrophil L-selectin expression (Figure 6 A). In contrast, IL-1R and TNFR:Fc given in combination, but not anti-CINC-1 antibody, blocked the LPS-induced upregulation CD11b/c (Figure 6 B). None of the antagonists affected circulating cell numbers or differentials (data not shown).

Discussion

Glucocorticoid suppression of the immune response to infection was first documented over sixty years ago. In a paper which appeared in 1932, Dr. Harvey Cushing described the syndrome, which would later bear his name, of hypercortisolism, and in this manuscript reported an increased susceptibility to infection in associate with this syndrome (28). The first report of clinical use of glucocorticoids by Hench et al. (29) for suppression of aberrant immune responses in diseases such as rheumatoid arthritis appeared in the literature in 1950, and was soon followed by case reports and animal studies which showed that administration of exogenous glucocorticoid for the treatment of disease was associated with a wide variety of side effects, including enhanced susceptibility to infection (30, 31). Despite this well-established effect of glucocorticoids, the mechanisms by which glucocorticoids suppress the immune response to infection have not been fully elucidated.

In the present studies, we have examined the effects of glucocorticoids on the earliest stages of leukocyte recruitment (i.e., rolling and adhesion) in response to bacterial LPS and have also examined the role of cytokines in LPS-induced leukocyte recruitment. Pretreatment of rats with DEX (18 hours or 30 min) dramatically reduced LPS-induced leukocyte rolling and adhesion in mesenteric postcapillary venules, and also inhibited LPS-induced changes in circulating leukocyte phenotype (i.e., L-selectin shedding, CD11b/c integrin upregulation). The data presented herein concerning the ability of DEX to inhibit LPS-induced changes in leukocyte rolling and adhesion, and in leukocyte phenotype, give new insight into the means by which glucocorticoid block LPS-induced leukocyte recruitment, and may also provide new insight into the protective role of glucocorticoids demonstrated in some animal models of sepsis. Although glucocorticoid administration in the absence of antibiotic treatment will enhance bacterial infection, some studies have demonstrated a decrease in mortality due to bacterial sepsis with glucocorticoid treatment(1). These effects are believed to be due to the ability of glucocorticoids to decrease expression of inflammatory mediators, such as TNF, IL-1 and IL-8, which contribute to the hemodynamic instability and organ failure associated with sepsis. Our data imply that

some of the protective effects of glucocorticoids in septic shock may also be mediated by inhibition of leukocyte recruitment responses and changes in circulating leukocyte phenotype which accompany the release of these cytokines. However. in blocking leukocyte recruitment responses. one also decreases the ability of leukocytes to kill bacteria. This may play a role in the lack of efficacy in human studies of high dose glucocorticoids for treatment of sepsis. as one of the complications of glucocorticoid therapy is secondary infection (32-34).

We hypothesized that the mechanism by which DEX inhibits leukocyte rolling and adhesion, as well as changes in leukocyte phenotype in our model. is via inhibition of cytokine and chemokine production and/or release. This hypothesis is supported by our findings that antagonists (i.e., soluble receptor or antibodies) to specific cytokines (IL-1 and TNF) and chemokines (CINC-1) effectively mimicked DEX effects on LPS-induced leukocyte rolling and adhesion. and on changes in circulating neutrophil phenotype. Administration of IL-1R and TNFR:Fc together. and in combination with the anti-CINC antibody. resulted in values for leukocyte rolling and adhesion which were not significantly different from values seen in DEX treated rats. Further support for this hypothesis is provided by data from previous studies which have found DEX to be ineffective in decreasing leukocyte rolling and adhesion in response to direct tissue stimulation with exogenously applied mediators (35-37). For instance. three different studies have reported that DEX does not decrease leukocyte rolling or adhesion in response to tissue stimulation with chemotactic agents such as leukotriene B4. FMLP. or platelet activating factor. although DEX does decrease transmigration of adherent leukocytes (35-37). The exception to this is a recent report by Tailor et al (37) in which DEX partially inhibited leukocyte adhesion in response to IL-1ß.

The major difference between our study and these previous studies is the type of stimulus utilized. In each of these studies (35-37), the microcirculatory tissue was directly stimulated with inflammatory mediators. while we stimulated with LPS. a substance known to induce inflammatory mediator synthesis. As such. our model may be more indicative of the normal tissue response to pathogens. where endogenous mediators are produced. This type of model

allows for the study of glucocorticoid effects on the production of inflammatory mediators, not just their effects on responses to these mediators. Interestingly, this is supported by data from very early intravital microscopy studies examining these same glucocorticoid effects (38-40). In the 1950's several groups noted that administration of glucocorticoids decreased leukocyte adhesion to the vascular endothelium in various models of inflammation including thermal injury, tuberculosis infection, and "serum sickness" (38-40). Though not known at the time, inflammation in these models relied on production of endogenous inflammatory mediators, and similar to our findings, glucocorticoids were very effective in inhibiting leukocyte-endothelial interactions under these conditions.

The most direct means to test whether glucocorticoids exert their effects on leukocyte recruitment by inhibiting cytokine and/or chemokine production would be to measure levels of these mediators in the mesenteric tissues. Unfortunately, the mesentery superfusion model makes this very difficult, as the superfusion buffer dilutes released cytokine by several hundred fold. Additionally, experiments in which the effects of DEX on exogenously administered cytokines, such as IL-1 and TNF, are examined may give some insight into the mechanism of these DEX effects, but these experiments are complicated by the ability of cytokines, particularly IL-1 and TNF, to induce release of other inflammatory mediators. For instance, in the case of the inhibitory effects of DEX on IL-1ß-induced leukocyte adhesion observed by Tailor et al. (37), it is possible that DEX inhibited leukocyte adhesion not by directly altering IL-1 effects, but by inhibiting IL-1-induced production of CINC-1, as IL-1 is the most potent stimulus for production of this chemokine. Similarly, LPS-induced TNF is believed to play a role in LPS-induced IL-1 production, which is itself DEX inhibitable.

One surprising outcome of the present studies, which also warrants further study, is the rapidity with which DEX affected the immune response to LPS. The primary means by which glucocorticoids mediate their actions is through regulation of gene expression (41-43), and numerous genes involved in metabolism, immunological responses, and inflammation, including the genes for IL-1, TNF and CINC-1, are known to be glucocorticoid-sensitive. As gene

transcription is the primary mechanism of action for glucocorticoids, the time course for glucocorticoid effects has been felt to be over the course of several hours. Thus, the majority of in vivo and in vitro experiments examining glucocorticoid effects have looked at glucocorticoid actions after prolonged (>4 hrs) treatment. In the present study we demonstrate that a single subcutaneous injection of DEX 30 min prior to LPS challenge was extremely effective in inhibiting LPS-induced leukocyte rolling and adhesion, and also L-selectin shedding and CD11b/c integrin upregulation when examined 1-2.5 hrs later. As the mechanism for these LPS affects appears to involve production of IL-1, TNF and CINC-1, these data imply that DEX may alter production and/or release of these inflammatory mediators more rapidly than previously believed. Further studies more closely examining the time course of glucocorticoid effects on gene regulation and inflammatory mediator production are necessary to determine if the mechanisms of glucocorticoid action are the same during prolonged verses acute treatment.

The data from our studies utilizing cytokine and chemokine antagonists, beyond their relevance to antiinflammatory mechanisms of glucocorticoids, also provide substantial new insight into the more basic mechanisms of LPS-induced leukocyte recruitment. Though LPS-induced cytokine generation and the role of these cytokines in the development of shock associated with bacterial sepsis in animals and man is well established (13-15), our data extend these findings by demonstrating the microvascular and systemic effects of these cytokines on leukocyte recruitment responses and leukocyte adhesion molecule expression. Superfusion of a single loop of mesentery with LPS resulted in sufficient cytokine production, either systemically or in the local mesenteric environment, to facilitate significant increases in leukocyte-endothelial interactions within 30 min of exposure to LPS. The rapidity with which these cytokines affected changes in leukocyte-endothelial interactions in vivo is in sharp contrast to the majority of in vitro studies in which several hours of endothelial cell stimulation with these cytokines is necessary to induce alterations in leukocyte adhesion responses (44).

As we have previously demonstrated that the changes in rolling and adhesion in this model system are mediated in large part by P- and L-selectin (21), these data imply that there is a

rapid change in the expression and/or function of these molecules in response to LPS-induced cytokine production. The fact that endothelial cells can be rapidly induced to express P-selectin on their surface is not surprising, as P-selectin is stored in the endothelial cell and is rapidly translocated to the endothelial surface in response to various stimuli, including histamine, leukotriene C4, and thrombin (45). What is not clear, however, is whether LPS or cytokines can directly regulate P-selectin expression, particularly in vivo. The ability of LPS to directly induce rapid P-selectin expression remains poorly defined (46, 47) and TNF has not been demonstrated to rapidly (30-60 min) upregulate P-selectin expression (48). Additionally, the contribution of cytokines in the maintenance of surface P-selectin expression following acute translocation has not been examined. In the case of L-selectin, leukocyte rolling mediated by L-selectin requires induction of the L-selectin ligand on endothelial cells. While both LPS and cytokines have been demonstrated to upregulate an as yet unidentified endothelial ligand for L-selectin (49), the time course of this action has not been examined. Our data imply that unlike E-selectin, the ligand for L-selectin may be upregulated within minutes after exposure to LPS-induced cytokines.

LPS-induced cytokines were also found to modulate adhesion molecule expression on circulating leukocytes in this system. Here we show that superfusion of a single loop of bowel with LPS resulted in significant alterations in L-selectin and CD11b/c integrin expression on circulating neutrophils. Following 2 hours of LPS superfusion, ~50% of circulating neutrophils no longer expressed detectable levels of L-selectin, while the circulating neutrophil population as a whole had increased CD11b/c integrin expression. Administration of IL-1R and TNFR:Fc completely inhibited these changes, while anti-CINC polyclonal antibody inhibited LPS-induced L-selectin shedding. These data indicate that cytokines generated by local bacterial infection may alter leukocyte recruitment response, not only at the site of infection, but also at distal tissue sites, as circulating neutrophils which lack L-selectin, and perhaps other selectin ligands, would be less able to interact with the endothelium and therefore less likely to be recruited out of the circulation.

As noted above, the apparent rapidity with which these cytokines are generated and influence leukocyte-endothelial interactions is of significance. All three mediators had significant effects on leukocyte rolling and adhesion within the 2 hour time course, although there were definite differences in time course of expression and function of each mediator. For instance, administration of TNFR:Fc was effective in inhibiting LPS-induced leukocyte rolling and adhesion by the earliest time point (30 min), while effects of IL-1R were not significant until 90 min. These data are consistent with previous in vivo studies demonstrating rapid, differential LPS-induced cytokine production (13-15). For example, Chensue et al. (15), utilizing a mouse model of endotoxemia in which LPS (80 μg) was given intraperitoneally, demonstrated by both immunohistochemistry and by biological assay that TNF and IL-1 were rapidly produced by mononuclear type cells in the liver and released into the circulation. TNF levels were maximal at one hour after introduction of LPS and rapidly decreased after this time point, while induction of IL-1ß generation was delayed, not reaching maximal levels until 6 hours after introduction of LPS, although present by 1 hour. Intravenous infusion of LPS resulted in similar findings in man (13, 14). Furthermore, the fact that simultaneous blockade of both cytokines was necessary to maximally inhibit leukocyte rolling and adhesion highlights the possible requirement for antagonism of multiple mediators in order to achieve the greatest anti-inflammatory effect.

The time course of CINC-1 production observed in our model is also consistent with previous data. Dolecki et al. (18), reported that mRNA for CINC-1 is detectable within 15 min of cell stimulation with IL-1, TNF and LPS in vitro, and protein is released within 1-2 hours. Although all three stimuli resulted in some increase in CINC-1 production, IL-1 was the most potent stimulus for CINC-1, with LPS being the second most potent and TNF the least potent. In our studies, CINC-1 was not found to play a significant role in early leukocyte rolling and adhesion (30 min), but was important at all later time points. This delayed time course for CINC-1 function may indicate that its production is downstream of, and thus mediated by, cytokine production in our model. Similarly, the fact that administration of the anti-CINC antibody was just as effective as administration of both cytokine antagonists in inhibiting

leukocyte rolling and adhesion at 60, 90 and 120 min indicate that one of the primary mechanisms by which cytokines may induce leukocyte endothelial interactions is through induction of this chemokine.

Interestingly, the data presented herein demonstrating the ability of the anti-CINC-1 antibody to block leukocyte rolling is the first direct evidence that the chemokine CINC-1 may play a role in mediating leukocyte rolling as well as adhesion. The CINC family of chemokines (i.e., CINC-1, CINC-2a, CINC-2b, CINC-3), which are most closely homologous to human or murine gro proteins, are similar in function to IL-8 in that they appear to function as neutrophil-specific chemoattractants (18-20). Recombinant CINC-1 has been demonstrated to induce neutrophil recruitment and to increase leukocyte adhesion and transmigration in vivo (50), but a role for CINC-1 in leukocyte rolling has not been established. In the present studies, an anti-CINC-1 antibody blocked both rolling and adhesion in response to LPS, indicating that CINC-1 may induce leukocyte rolling responses. This is supported by data from Harris et al (51), in which they show a role for the selectins, primarily P-selectin and L-selectin, in CINC-1 induced neutrophil recruitment. These authors contend that P-selectin expression in their model is the result of CINC-1 induced histamine release (51), however based on studies with histamine (H1) antagonists, we have been unable to demonstrate a role for histamine in our model (unpublished observation).

In conclusion, the data presented demonstrate that the glucocorticoid DEX inhibits LPS-induced leukocyte recruitment by inhibiting the earliest phases of leukocyte recruitment, leukocyte rolling and adhesion, and that glucocorticoids also inhibit changes in the adhesion phenotype of circulating neutrophils. DEX appears to mediate these effects through inhibition of inflammatory mediator release, especially the IL-1, TNF and CINC-1. These data provide new insight into the mechanisms by which glucocorticoid therapy alters neutrophil recruitment responses to LPS.

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Footnotes

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[2]Correspondence to:Bruce S. Bochner, M.D., Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, Phone: (410) 550-2131. FAX: (410) 550-2130 email: bbochner@welchlink.welch.jhu.edu

[3] Abbreviations used in this paper: CINC-1, cytokine-induced neutrophil chemoattractant-1; DEX, dexamethasone; IL-1R, soluble IL-1 receptor; TNFR:Fc, soluble TNF receptor.

Figure Legends

Figure 1. DEX inhibited LPS-induced leukocyte rolling (A) and adhesion (B). Superfusion of the rat mesentery with LPS (1 μg/ml) resulted in rapid increases in leukocyte rolling and adhesion. Pretreatment of rats with DEX (0.5 mg/kg) 18 hrs prior to initiation of LPS superfusion completely inhibited LPS-induced leukocyte rolling and adhesion (n = 6). Pretreatment with DEX for only 30 min also significantly decreased leukocyte rolling and adhesion (n = 6). The non-glucocorticosteroid hormone testosterone (TESTOST. 0.5 mg/kg) given as a control did not effect LPS-induced leukocyte-endothelial interactions when given either 18 hrs (n = 4) or 30 min (n =2) prior to LPS superfusion (data is combined in figure for n = 6 as results were similar). * indicates values for 18 hr and 30 min DEX treated rats which are significantly ($p<0.05$) different from the LPS + PBS condition.

Figure 2. DEX effects on circulating leukocyte differentials. Pretreatment of rats with DEX for 18 hrs. but not 30 min. resulted in a significant (*$p<0.05$) decrease in the percentage of circulating lymphocytes and an increase in the percentage of circulating neutrophils at baseline when compared to values from LPS + PBS animals. Lymphocytes and neutrophil percentages in rats pretreated with DEX for 18 hrs remained significantly different from values in the other treatment groups after 120 min of LPS superfusion. (n = 4-6 for all groups).

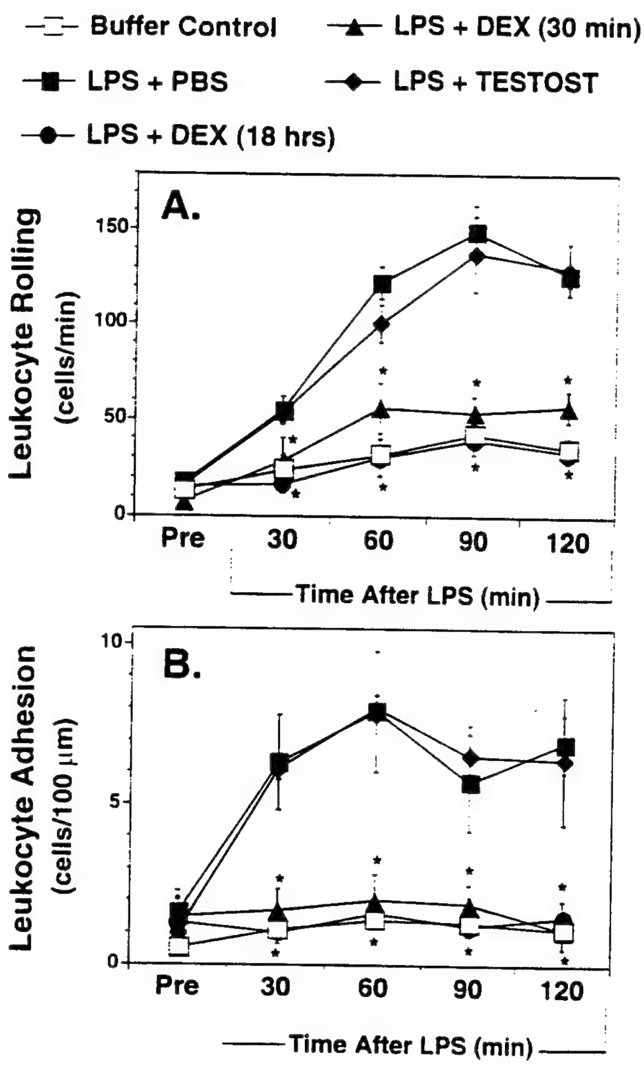
Figure 3. DEX effects on LPS-induced changes in neutrophil expression of L-selectin (A) and a shared CD11b/c epitope (B). Superfusion of a single loop of rat mesentery with LPS for 2 hrs resulted in a significant decrease in the percent of neutrophils expressing L-selectin and a significant increase in CD11b/c expression as compared to baseline values. Pretreatment of rats with DEX for 18 hrs or 30 min completely inhibited in vivo LPS-induced changes in leukocyte L-selectin and CD11b/c integrin expression. *indicates values after LPS which were significantly ($p<0.05$) different from pre-LPS (0 min) values (n = 4-5). (C) DEX does not inhibit

ex vivo LPS-induced L-selectin shedding. Stimulation of rat whole blood with LPS (30 min. 37°C) resulted in concentration dependent decreases in L-selectin expression on neutrophils from control and DEX (18 hrs and 30 min) treated rats. Neutrophils from rats pretreated with DEX for 18 hrs displayed significantly enhanced L-selectin shedding in response to LPS in vitro (n = 5). * indicates values significantly (p<0.05) different from control animals.

Figure 4. Role for IL-1 and TNF in LPS-induced leukocyte rolling (A) and adhesion (B). Intravenous infusion of TNFR:Fc (100 µg/kg, ten minutes prior and 60 min after starting LPS superfusion. n = 5) significantly inhibited leukocyte rolling at all time points. while administration of IL-1R (100 µg/kg. ten minutes prior and 60 min after starting LPS superfusion. n = 5) significantly inhibited leukocyte rolling only at later (90 and 120 min) time points. TNFR:Fc significantly inhibited leukocyte adhesion at 30 and 120 min. while IL-1R only inhibited adhesion at the latest time point. Simultaneous infusion of IL-1R and TNFR:Fc (n = 5) significantly inhibited LPS-induced increases in leukocyte rolling and adhesion at all time points. * indicates values significantly (p<0.05) different from the LPS + PBS condition.

Figure 5. Role for CINC-1 in LPS-induced leukocyte rolling (A) and adhesion (B). Infusion of anti-CINC-1 polyclonal antibody (2 mg/rat. n = 5) did not inhibit early (30 min) leukocyte rolling or adhesion. but significantly inhibited leukocyte rolling and adhesion at all later time points. * indicates values significantly (p<0.05) different from the LPS + PBS condition.

Figure 6. Effects of IL-1R and TNFR:Fc. or anti-CINC-1 antibody, on LPS-induced alterations of circulating neutrophil phenotype. Pretreatment of rats with IL-1R and TNFR:Fc in combination prevented in vivo LPS-induced L-selectin shedding (A) and CD11b/c integrin upregulation (B) (n = 3). Pretreatment of rats with an anti-CINC-1 polyclonal antibody inhibited LPS-induced L-selectin shedding, but did not prevent upregulation of CD11b/c (n = 5). * indicates values significantly (p<0.05) different from pre-LPS (0 min) values.



Buffer Control
LPS + DEX (30 min)
LPS + PBS
LPS + TESTOST
LPS + DEX (18 hrs)
A.
Leukocyte Rolling
(cells/min)
150
100
50
0
Pre
30
60
90
120
Time After LPS (min)
B.
Leukocyte Adhesion
(cells/100 μm)
10
5
0
Pre
30
60
90
120
Time After LPS (min)

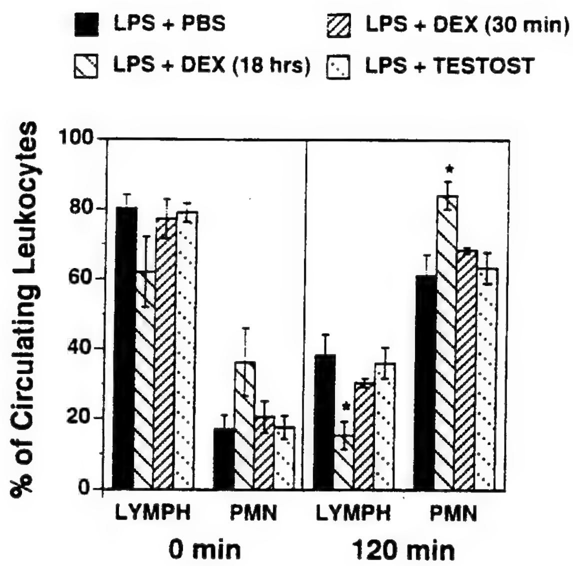
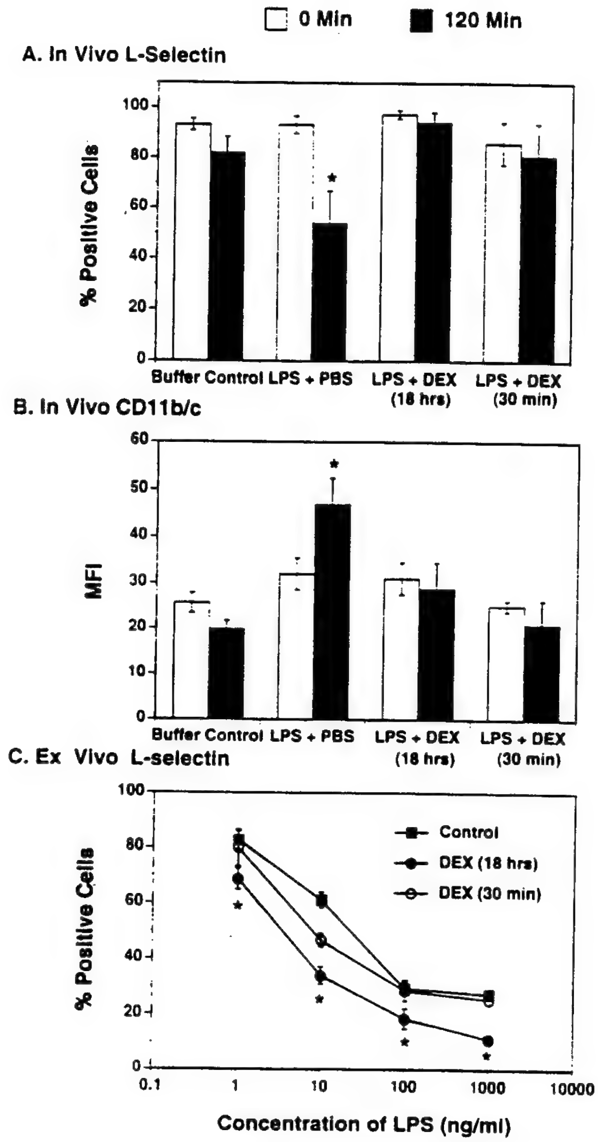
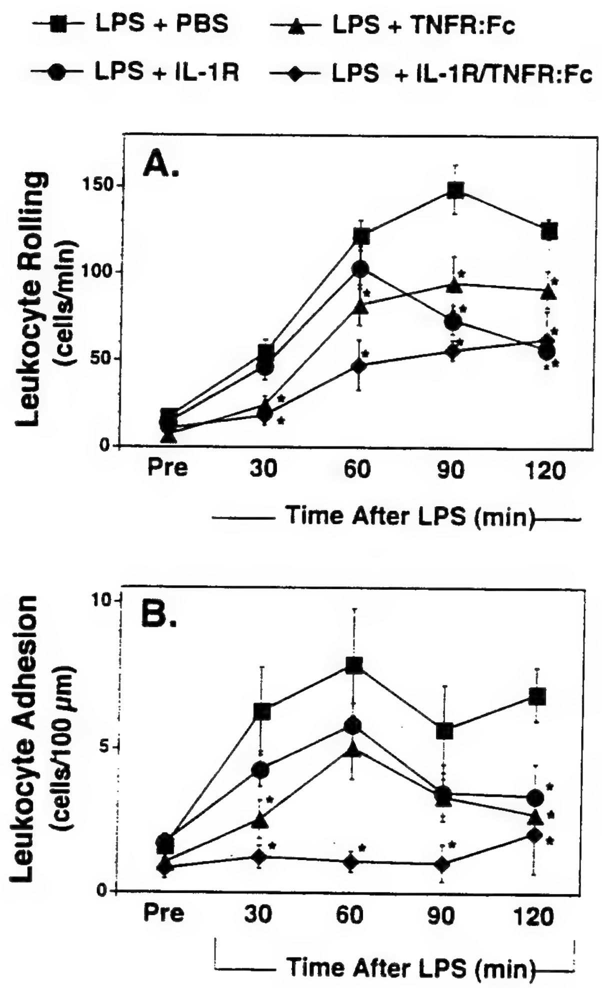

LPS + PBS
LPS + DEX (30 min)
LPS + DEX (18 hrs)
LPS + TESTOST
% of Circulating Leukocytes
100
80
60
40
20
0
*
*
LYMPH
PMN
LYMPH
PMN
0 min
120 min

Fig. 2

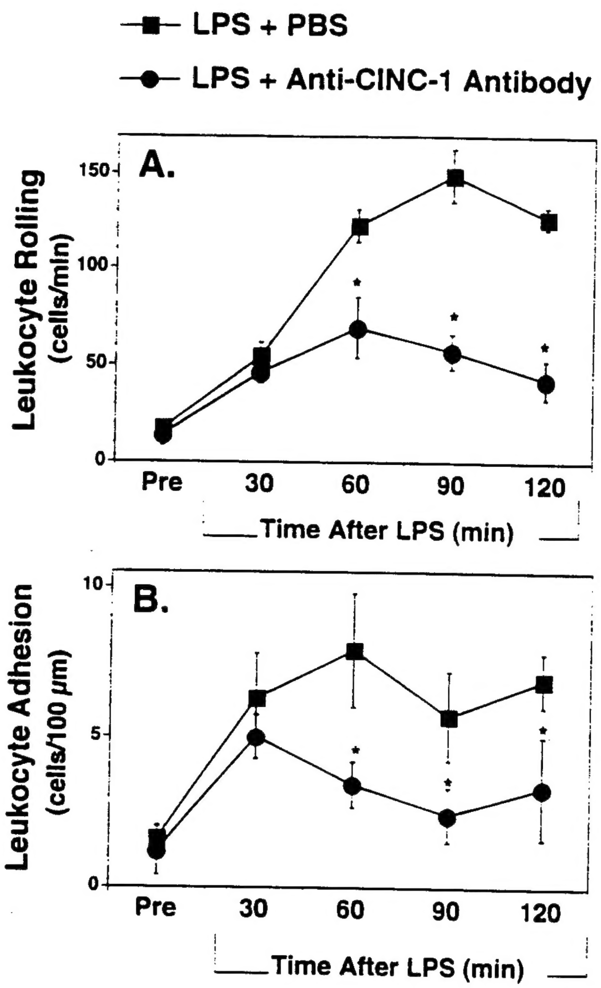


0 Min
120 Min
A. In Vivo L-Selectin
% Positive Cells
Buffer Control
LPS + PBS
LPS + DEX (18 hrs)
LPS + DEX (30 min)
B. In Vivo CD11b/c
MFI
C. Ex Vivo L-selectin
Control
DEX (18 hrs)
DEX (30 min)
Concentration of LPS (ng/ml)



LPS + PBS
LPS + TNFR:Fc
LPS + IL-1R
LPS + IL-1R/TNFR:Fc
A.
Leukocyte Rolling (cells/min)
150
100
50
0
Pre
30
60
90
120
Time After LPS (min)
B.
Leukocyte Adhesion (cells/100 μm)
10
5
0
Pre
30
60
90
120
Time After LPS (min)

Fi. 4



LPS + PBS
LPS + Anti-CINC-1 Antibody
A.
Leukocyte Rolling (cells/min)
150
100
50
0
Pre
30
60
90
120
Time After LPS (min)
B.
Leukocyte Adhesion (cells/100 μm)
10
5
0
Pre
30
60
90
120
Time After LPS (min)

Fig. 5

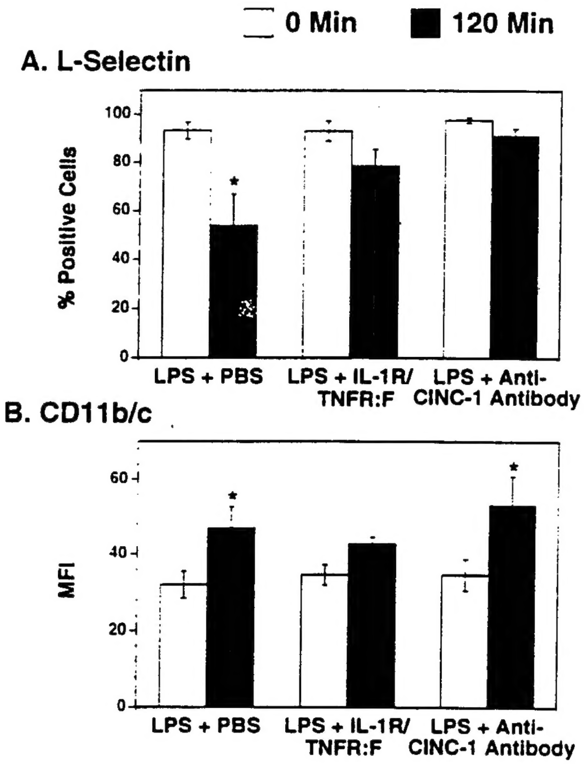

0 Min
120 Min
A. L-Selectin
% Positive Cells
100
80
60
40
20
0
*
LPS + PBS
LPS + IL-1R/ TNFR:F
LPS + Anti-CINC-1 Antibody
B. CD11b/c
MFI
60
40
20
0
*
*
LPS + PBS
LPS + IL-1R/ TNFR:F
LPS + Anti-CINC-1 Antibody

Fig. 6

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13. ABSTRACT *(Maximum 200 words)*
Critical progress has been made in the identification and characterization of cells and mediators involved in allergic inflammation. Accumulating evidence supports the importance of cell adhesion molecule expression as an initiating process in tissue inflammation. Despite progress made to date, much is still unknown about the exact mechanisms responsible for this inflammatory response. Scientists have been working to understand the selective cell recruitment operating in allergic disease with the hope of discovering therapeutic intervention strategies that will prevent the accumulation of unwanted cells in inflamed airways. Research has been directed at developing various approaches to generate specific antagonists. Some approaches under study interupt airway inflammation in its early stages during leukocyte-endothelial interactions. Other approaches inhibit cell recruitment at the endothelial wall. Many studies have been done, both <u>in vivo</u> and <u>in vitro</u>, and the advances that have been made suggest that these therapeutic interventions may be the keys to controlling and, possibly, curing asthma and allergic reactions.

14. SUBJECT TERMS
cell adhesion, allergic rhinitis, atopic allergic conditions, cell biology

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